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Bacterial degradation of dehydropolymers of coniferyl alcohol

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Abstract. A bacterial isolate identified as *Xanthomonas* sp. proved to be ligninolytic due to its ability to degrade ^{14}C-labeled dehydropolymers of coniferyl alcohol (DHP) and [14C]lignocellulose complexes from corn plants *(Zea mays)*. Several parameters of ligninolysis were evaluated and it was shown that resting cells degrade DHP as sole carbon source. Enhancement of DHP degradation in the presence of ferulic acid or water-soluble fractions of DHP or of dioxane lignin from wheat was demonstrated. It is shown that a dissociation of DHP takes place during incubation in the absence of the bacteria which is reflected in a shift of DHP to lower molecular weight fractions. Bacterial degradation of [14C] DHP results in the release of $^{14}CO_2$ and in the incorporation of the ^{14}C-label into the biomass of the bacteria, as shown by chemical and biological methods.

Key words: Bacteria – Lignin degradation – Synthetic lignin – Natural lignin – Lignin-cellulose complex – $^{14}CO_2$ evolution – Ferulic acid – Permeation chromatography

Biological decomposition of lignin, the most abundant biopolymer in woody plants next to cellulose, is a very important process in the natural carbon cycle. Under natural conditions lignin degradation and transformation is brought about by mixed populations of fungi and bacteria. It is obvious that the ability to degrade lignin is not limited to the white rot fungi but extends also to soft rot fungi and to numerous bacteria (Crawford 1981), as has been shown by non-isotopic methods and by using ^{14}C-labeled lignin or lignin model compounds. Although there are indications that lignin degradation by bacteria takes place in a similar way to that by white rot fungi, as shown with *Streptomyces viridosporus* (Crawford et al. 1982), lignin degradation itself has not yet been fully elucidated. There is general agreement that synthetic lignin, a ^{14}C-labeled dehydrogenated polymer of coniferyl alcohol (DHP), is a valuable tool in studying lignin biodegradation processes. However, it is also admitted that DHP is not identical to natural lignin and care should be taken in drawing conclusions only from experiments with DHP.

Abbreviations. Bq, Becquerel, measure for radioactivity according to SI nomenclature; DHP, dehydropolymers of coniferyl alcohol; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; HPLC, high performance liquid chromatography; TCA, trichloroacetic acid; THF, tetrahydrofuran

In the following studies, lignin degrading bacteria were isolated as a result of a screening program. Physiological investigations on the degradation of DHP by a bacterium identified as *Xanthomonas* sp. will be reported.

Materials and methods

Preparation of lignin and dehydropolymers of coniferyl alcohol (DHP)

Dioxane lignin was extracted from dry wheat straw at ambient temperatures for 20 d and purified as described by Odier and Monties (1978).

Preparation of ^{14}C-labeled DHP's was performed from coniferyl alcohol according to the "Zutropf"-method (Freudenberg 1968). The synthesis of specifically ^{14}C-labeled coniferyl alcohol was carried out using published methods (Haider and Trojanowski 1975). Briefly, vanillin (ring-U-^{14}C) produced by Amersham-Buchler (Braunschweig, FRG) was reacted with malonic acid to form ferulic acid and then reduced to coniferyl alcohol. Analogous syntheses were performed with vanillin and [2-^{14}C]malonic acid to give [^{14}C-β]coniferyl alcohol. Ferulic acid, ^{14}C-labeled in the methoxy group, was kindly supplied by Dr. K. Haider (Braunschweig, FRG). The different [^{14}C]DHP's were purified by precipitating several times from dioxane into diethyl ether. In some cases (cf. Fig. 6) additional purification steps including treatment with n-butanol and precipitations with acetic acid (Faix et al. 1981) were performed. Only the butanol insoluble fraction of the DHP was used.

^{14}C-labeled lignocellulose complexes were prepared from corn plants *(Zea mays)* which were fed with specifically ^{14}C-labeled ferulic acid according to the procedure described by Haider and Trojanowski (1975). In order to remove ester bound ferulic acid, the plant material was treated with cold 1 M NaOH. Lignocellulose complexes, ^{14}C-labeled in C-γ or in the methoxy group, were kindly supplied by Dr. K. Haider (Braunschweig, FRG).

Isolation of ligninolytic bacteria

Soil suspensions in 0.9% NaCl were incubated in a mineral salts medium containing per litre: 0.4 g KH_2PO_4, 1.6 g K_2HPO_4, 0.5 g NH_4NO_3, 0.2 g $MgSO_4 \cdot 7\,H_2O$, 0.015 g $FeCl_3$, 0.5 mg $MnSO_4 \cdot H_2O$, 1 mg $ZnSO_4 \cdot 7\,H_2O$, 0.5 mg $CaCl_2 \cdot 2\,H_2O$, 1 mg $CuSO_4 \cdot 5\,H_2O$, 0.1 mg KCl, 0.5 mg H_3BO_3, adjusted to pH 7 with HCl. This medium was supplemented with dioxane lignin from wheat (0.08% w/v)

as the sole carbon source except for the fact that the medium contained 0.1% glucose as starting carbon source during the first incubation. After several transfers of shaken cultures at 30°C, aliquots were serially diluted and plated on agar having the same composition as the medium above. Dioxane lignin from wheat dissolved in 0.2 M NaOH was mixed with the melted agar. The medium was neutralized by adding equivalent amounts of HCl before solidifying. After incubation for several days replica printings were made onto agar prepared from a complete medium (yeast extract 10 g, glucose 10 g, KH_2PO_4 1 g and $MgSO_4 \cdot 7\,H_2O$ 0.5 g/l). Developing colonies were transferred into liquid complete medium, incubated for 24 h at 30°C, centrifuged, washed free from this medium and suspended in 0.9% NaCl. The following two tests to detect ligninolytic activity were performed in parallel: 30 ml of the mineral salts medium were supplemented either with 30 mg dioxane lignin from wheat or with [^{14}C]DHP (4.7 mg DHP/assay; the specific radioactivity of the [$^{14}C_{ring}$]DHP was 345 Bq/mg DHP). Lignins were dissolved in dimethylformamide (DMF) and added to the medium. Both assays were inoculated with ca. 30×10^9 bacteria each. After cultivation for one or two weeks the total amount of the unlabeled dioxane lignin in the assay and also in the uninoculated control was extracted with dioxane and determined quantitatively by measuring the absorption at 280 nm. During incubation with [^{14}C]DHP the release of $^{14}CO_2$ was followed by trapping CO_2 in NaOH as described by Haider and Trojanowski (1975). Cultures, positive with regard to both tests, were subcultured on agar with complete medium and single cell cultures were isolated. Homogeneity of the selected strains was tested by plating and microscopical observation followed by taxonomic determinations (Kern et al., unpublished). The bacterial strains were stored at −20°C in the mineral medium containing 50% glycerol.

Analyses of DHP degradation

Routine assays (30 ml) were performed in aerated shaking cultures. Some experiments were conducted under atmospheres with different pO_2. In such cases inoculated medium (15 ml) supplemented with [$^{14}C_{ring}$]DHP in 100 ml firmly closed flasks were evacuated and flushed with argon. By withdrawing part of the gas phase and replacing it with respective volumes of oxygen different partial pressures of O_2 were established. After termination of the experiments (14 d) the flasks were flushed with nitrogen and the evolved $^{14}CO_2$ was trapped with NaOH and measured as above.

In order to quantify the degradation of the [^{14}C]DHP, the released amounts of $^{14}CO_2$ were measured, the radioactivity in dioxane extracts from the terminated culture was determined and the dioxane unextractable radioactivity was calculated (Table 2) or determined by combustion. In all examined assays at least 96% of the total radioactivity was recovered. The attribution of the radioactivity remaining in dioxane extracted bacteria to different biopolymers was performed by means of a fractionation procedure according to Schmidt and Thannhauser (1945): hydrolysis in 0.2M NaOH at 37°C for 18 h, precipitation of DNA and proteins with 10% trichloroacetic acid (TCA, w/v) in the cold, differentiation between DNA and proteins by extraction of DNA with 5% TCA at 90°C. Preparation of ribosomal RNA from dioxane extracted bacteria was performed according to the procedure described by Kirby (1965) and as modified by Kern (1975).

The biological detection of the ^{14}C-label incorporated into the biomass of the bacteria after incubation with [^{14}C]DHP was accomplished as follows: a series of incubations of ligninolytic bacteria (10 ml) with known amounts of [$^{14}C_{ring}$]DHP was terminated by autoclaving. The residual radioactivity, consisting of undegraded [^{14}C]DHP and the label incorporated into the biomass of the bacteria, was calculated by subtraction of the amounts of ^{14}C released as $^{14}CO_2$ from the total administered radioactivity. This residual radioactivity in the terminated cultures was set as 100% for the subsequent incubation with the non-ligninolytic bacterium *Proteus vulgaris*. These bacteria were precultivated in a peptone meat extract medium (peptone 5 g, meat extract 3 g/l, pH 7) for 24 h at 30°C; 15 ml of this culture were added to the autoclaved assays (10 ml) and incubated for 25 days with simultaneous determination of $^{14}CO_2$ released from the biomass of the ligninolytic bacteria. By using appropriate controls it could be shown that *Proteus vulgaris* was unable to mineralize either [^{14}C]DHP or lignin-related monomers such as [$^{14}C_{ring}$]ferulic acid.

Gel permeation chromatographic separations of [$^{14}C_{ring}$]DHP at different stages of degradation by the ligninolytic bacteria were performed on Sephadex LH-60 columns (length 151 cm, diameter 1.6 cm) using dimethylformamide (DMF) containing 0.1 M LiCl as eluant (Connors et al., 1980). The distribution of the ^{14}C-label in the effluents of the column was followed by liquid scintillation spectroscopy. The quantitative recovery of ^{14}C from the assays and also from the adequate uninoculated controls was performed by extraction with dioxane. The dioxane extracts were supplied with DMF, evaporated to a small volume and applied to the column. The LH-60 column was calibrated with polystyrene standards localized in the effluents at 271 nm.

In order to demonstrate dissociation of the [^{14}C]DHP during incubation in the uninoculated medium, [^{14}C]DHP further purified according to Faix et al. (1981) was recovered after the indicated time (cf. Fig. 6) of incubation, freeze-dried and acetylated with a mixture of acetic anhydride and pyridine (1:1, v/v) for 24 h at 50°C. After the reaction the acetylation reagents were removed by evaporation, the residue was dissolved in tetrahydrofuran (THF), filtered and analyzed by HPLC on 5 microgel columns (Chrompak, Netherlands B.V.) coupled with increasing pore size (5-, 10-, 50-, 10^2 and 10^3 nm columns, length 25 cm each, inner diameter 7.7 mm, outer diameter 9.5 mm) under similar conditions as described by Faix et al. (1981). The chromatograph (Hewlett Packard 1080 B), equipped with a fraction collector, allowed the determination of radioactivity in the effluents and the correlation of this with the UV-absorption, scanned at 280 nm.

Results

The strategy pursued in isolating the bacteria required direct detection of ligninolytic activity as the most important step. The isolates in question were tested for their ability to degrade lignin by using [^{14}C]DHP preparations and measuring the released $^{14}CO_2$ and also by incubating in the presence of unlabeled dioxane lignin from wheat. In the latter case

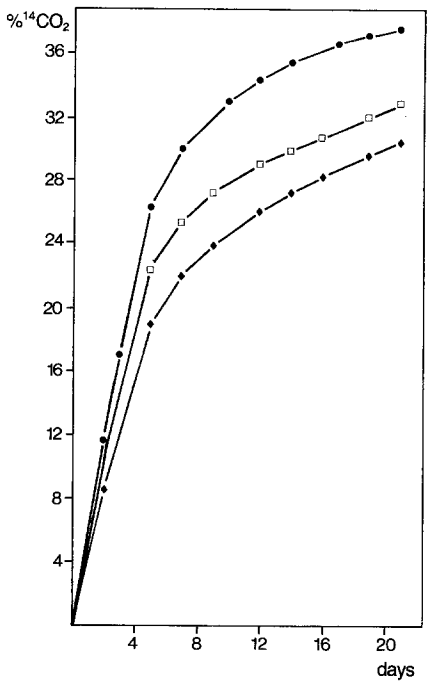

Fig. 1. Biooxidation of different ^{14}C-DHP's by *Xanthomonas* sp.: ◆——◆ [^{14}C-β]DHP; □——□ [$^{14}C_{ring}$]DHP; ●——● [$O^{14}CH_3$]DHP

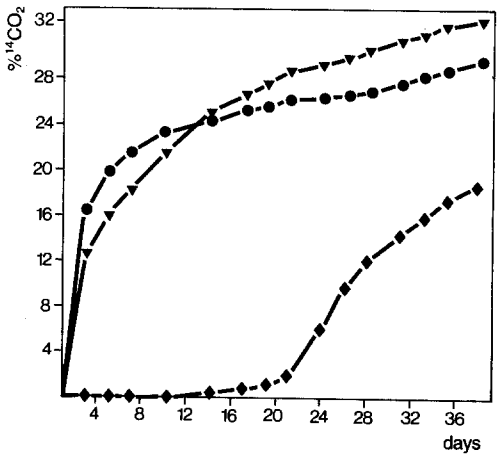

Fig. 2. Mineralization of [$^{14}C_{ring}$]DHP by the bacteria: ●——● release of $^{14}CO_2$ under standard conditions; ▼——▼ incubation in the presence of 0.8 mM ferulic acid; ◆——◆ incubation in the presence of 1.6 mM ferulic acid. Each experiment was started with the same number of bacteria

the decrease in lignin was determined spectroscopically (A_{280}). By plating on agar it could be shown that many isolates with a high ligninolytic activity towards lignin from wheat (up to 35% degradation within 14 d) consisted of different types of bacteria. With increasing homogeneity of the selected cell cultures ligninolytic activity against dioxane lignin decreased. For further investigations only one of the isolated bacterial strains was used. It effects a degradation of dioxane lignin of only about 5% within 14 d but showed no loss of activity towards [^{14}C]DHP's within the last two years. This strain was identified as *Xanthomonas* sp. (Kern et al., unpublished).

Influence of different physical and physiological parameters on the bio-oxidation of ^{14}C-labeled DHP

As described under methods, the bacteria were grown in a complete medium and then, after washing transferred to the mineral salts medium containing [^{14}C]DHP as the sole carbon source. Under standard conditions an extensive oxidation of the [^{14}C]DHP preparations could be detected by measuring the released $^{14}CO_2$. As can be seen from Fig. 1, mineralization starts in all cases after two days of incubation and [^{14}C]DHP, labeled in the methoxy group, is degraded to a higher extent than the corresponding [^{14}C-β]- or [$^{14}C_{ring}$]DHP's. The more ready oxidation of the methoxy group has also been reported by other authors (Haider and Trojanowski 1980).

The influence of various physiological and physical parameters which are important in microbial lignin degradation (Crawford 1981; Kirk 1981) was investigated for the bacterial oxidation of [^{14}C]DHP. The results obtained are given in Table 1. Obviously, the bacteria can utilize DHP as sole carbon source and in contrast to many other lignin degrading organisms (Crawford 1981) there was no indication of co-oxidation. The bacteria are devoid of cellulolytic activities. When incubations in the presence of easily accessible carbon sources such as glucose were performed, cell growth began and at the same time mineralization of [^{14}C]DHP ceased. It was shown that the bacteria are able to utilize some lignin related compounds such as coniferyl alcohol, ferulic acid, vanillic or protocatechuic acid as the sole carbon source for growth. In the presence of these substances the synthesis of protocatechuate oxygenase was enhanced, indicating that ring cleavage proceeds via the ortho-mechanism (3,4 cleavage). During incubation in the presence of [$^{14}C_{ring}$] coniferyl alcohol the bacteria performed first the oxidation to coniferyl aldehyde, ferulic and vanillic acid. As soon as the latter metabolite was produced, ring cleavage occurred and further oxidations led to the release of $^{14}CO_2$ (Kern et al., unpublished). In Fig. 2 the mineralization of DHP by the bacteria in the absence or presence of different amounts of ferulic acid is illustrated. Compared to the incubation in the absence of ferulic acid, only a slight and temporary limited depression in mineralization resulted in the presence of low amounts of this aromate (0.8 mM), whereas a higher concentration of ferulic acid (1.6 mM) caused a long delay in the mineralization of DHP. In accordance with the growth experiments with glucose, mentioned above, it could be shown by cell counting that the onset in the release of $^{14}CO_2$ reflected the termination of cell growth. No evidence for any increase in the cell number could be obtained in incubations in the absence or presence of low amounts of ferulic acid (0.8 mM). In the presence of higher amounts of ferulic acid (1.6 mM), however, cell growth was detectable which ceased when the release of $^{14}CO_2$ occurred. Apparently, the mineralization of [^{14}C]DHP is effected by resting cells as could be concluded from experiments with an inhibitor of DNA synthesis, nalidixic acid (Table 1).

As can be seen from Table 1, mineralization of [^{14}C]DHP occurred to almost the same extent when incubations were conducted under air or under 100% oxygen. Due to the very different pO_2 in these experiments a series of incubations were carried out under strictly anaerobic conditions and at different partial pressure of oxygen under otherwise identical conditions. It was found that the release of $^{14}CO_2$ already took place at $pO_2 = 0.1$ bar at the

Table 1. Dependence of bacterial degradation of [$^{14}C_{ring}$]DHP upon different physical and physiological parameters

Parameter	Conditions	% ^{14}C released as $^{14}CO_2$ (d)
Incubation under air	normal medium	29.5 (20 d)
Incubation under 100% oxygen	normal medium	29.3 (20 d)
Incubation under air	0.9% NaCl	23.7 (21 d)
Incubation under 100% oxygen	0.9% NaCl	21.0 (21 d)
Incubation at different pH:		
pH range 3.0–5.5	normal medium (diluted)	>25.0 (20 d)
pH range 6.0–6.5	normal medium (diluted)	28.3 (20 d)
pH range 7.0–8.0	normal medium (diluted)	>25.0 (20 d)
Carbon sources:		
cellulose (MN 300)	1% (w/v), normal medium	28.9 (20 d)
glucose	0.5–2% (w/v), normal medium	2.4 (20 d)
Nitrogen supply:		
NH_4NO_3	0–1 g NH_4NO_3/l, normal medium, incubation under air	29.5 (21 d)
Inhibition of DNA synthesis by nalidixic acid	100 µg/ml, normal medium	26.6 (21 d)

Incubations of the bacteria were performed under shaking conditions. If not indicated, normal medium was used under standard conditions under air

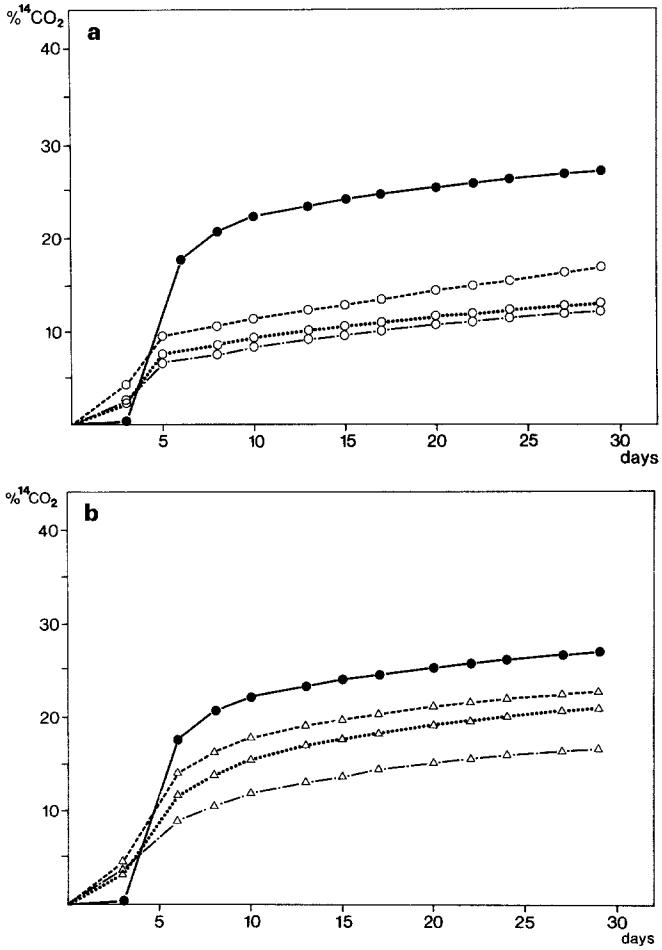

Fig. 3. a Mineralization of [$^{14}C_{ring}$]DHP by the bacteria in the presence of increasing amounts of homologous (unlabeled) DHP: ●——● carrier-free [$^{14}C_{ring}$]DHP; ○– – –○ addition of 9 mg DHP; ○ · · · · ○ addition of 18 mg DHP; ○ · – · ○ simultaneous addition of 27 mg DHP. In each assay (triplicates for each concentration of DHP) the same amount of [$^{14}C_{ring}$]DHP and the same number of bacteria were used. **b** Mineralization of [$^{14}C_{ring}$]DHP by the bacteria in the presence of increasing amounts of heterologous (unlabeled) lignin (dioxane-lignin from wheat): ●——● carrier-free [$^{14}C_{ring}$]DHP; △– – –△ addition of 9 mg lignin; △ · · · · △ addition of 18 mg lignin; △ · – · △ simultaneous addition of 27 mg lignin. In each assay (triplicates for each concentration of lignin) the same amount of [$^{14}C_{ring}$]DHP and the same number of bacteria were present

same level as under air (pO_2 = 0.2 bar) or even under an atmosphere of 100% oxygen. No indication for anaerobic mineralization of DHP could be observed.

Different action of the bacteria upon DHP and dioxane lignin from wheat

In the following the relation between the number of bacteria and the mineralization rate of DHP was investigated. It was possible to correlate the number of living cells, counted by plating on agar with complete medium, with measurement of turbidity (A_{260}) of cells suspended in 0.9% NaCl and to determine the number of bacteria by both methods. It was found that 1.05×10^9 bacteria per ml (a total of 31.5×10^9 bacteria per assay with 30 ml) could convert at least 25% of the [^{14}C]DHP supplied (4.7 mg [$^{14}C_{ring}$]DHP with a specific radioactivity of 345 Bq/mg DHP) into $^{14}CO_2$ within 20 d. In the following experiments equal numbers of bacteria were incubated with increasing amounts of DHP. In this case [$^{14}C_{ring}$]DHP with high specific radioactivity (13.9 kBq/mg DHP) was used and by adding unlabeled DHP different specific radioactivities were established. As indicated in Fig. 3a, each assay contained 0.12 mg of the DHP with high specific radioactivity (equalling 1.67 kBq) mixed with increasing amounts of unlabeled DHP. According to the amounts of applied DHP a depression in the release of $^{14}CO_2$ was measured approaching a final level. In corresponding experiments the [^{14}C]DHP (also 0.12 mg per assay) was mixed with increasing amounts of heterologous lignin (dioxane lignin from wheat) instead of DHP and assayed under otherwise identical conditions as above. As illustrated in Fig. 3b, the depression in the release of $^{14}CO_2$ was comparatively lower and a pronounced dependence on the administered amounts of lignin could be observed. Since, as mentioned above, the degradation rate of dioxane lignin by the bacteria compared to DHP is very low, these results can be considered as a reflection of different affinities by the bacteria because structural dissimilarities between the two types of lignin are probably decisive; apparently only slight competition between both lignins occurs.

These two series of experiments (Fig. 3a, b) have the following observations in common: compared with the

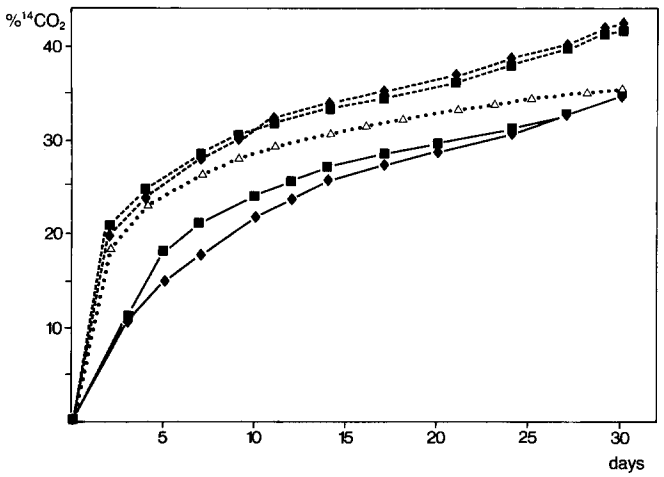

Fig. 4. Mineralization of [$^{14}C_{ring}$]DHP by the bacteria: ■——■ release of $^{14}CO_2$ under standard conditions; ◆——◆ in the presence of ferulic acid (0.8 mM); ■– – –■ after preincubation of the bacteria with ferulic acid (2.6 mM) for 3 d followed by the transfer into the medium without ferulic acid; ◆– – –◆ preincubated bacteria (2.6 mM ferulic acid) were incubated with [^{14}C]DHP in the presence of 0.8 mM ferulic acid; △· · · △ preincubation of the bacteria with the water soluble fraction of unlabeled DHP (4 d). Equal numbers of bacteria were used in each experiment

course of the mineralization of the carrier-free [^{14}C]DHP (0.12 mg [^{14}C]DHP per assay), there is a depression in the release of $^{14}CO_2$ in both cases if corresponding amounts of DHP or lignin are added; however, it is apparent that in both series an earlier onset in the mineralization of [^{14}C]DHP can be observed. This seems to be dependent on the presence of higher amounts of DHP or lignin in the medium and a tentative explanation for this fact could be that water-soluble fractions of both DHP and lignin have inducing or at least stimulating effects on the ligninolytic activity of the bacteria.

Enhancement of DHP degradation by ferulic acid or water-soluble fractions of lignins

In order to support this assumption, further experiments were conducted. Firstly the question was raised whether or not lignin-related monomers such as ferulic acid could effect higher ligninolytic activities of the bacteria. As mentioned above, the intracellular content of protocatechuate oxygenase can be increased by incubating the bacteria with ferulic acid. As shown in Fig. 4, a preincubation of the bacteria with 2.6 mM ferulic acid caused an increase in the ligninolytic activity of the bacteria which moreover started earlier than in the corresponding control. The release of $^{14}CO_2$ took place at almost the same extent if the preincubated cells (2.6 mM ferulic acid) were assayed in the presence of 0.8 mM ferulic acid. In contrast to this, the mineralization of DHP showed a temporarily limited depression if untreated bacteria were incubated in the presence of 0.8 mM ferulic acid (cf. also Fig. 2).

Secondly it was investigated whether the stimulating effect could also be achieved with the water-soluble fractions of DHP as postulated. For this purpose, unlabeled DHP was incubated for 4 days under experimental conditions in the absence of the bacteria, the insoluble DHP was separated by centrifugation and the supernatant used for a preincubation of the bacteria in the same way as with ferulic acid described above. A stimulating effect on the bacteria was clearly observed in this case (Fig. 4). Experiments with [^{14}C]DHP are also in agreement with these aspects. In this context, [^{14}C]DHP was first incubated for 5 days under experimental conditions without the bacteria and then, after centrifugation the sedimented DHP was offered to the bacteria in the usual way. In this case only 20% conversion to $^{14}CO_2$ was measured after 20 days of incubation, instead of about 26% as in the previous experiments, at otherwise similar kinetics. From these various experimental approaches it can be concluded that the bacteria can be stimulated in their ligninolytic activities towards DHP by ferulic acid or by water-soluble fractions of DHP and probably also of dioxane lignin (Fig. 3b).

Table 2. Distribution of ^{14}C-radioactivity in bacterial cultures with [$^{14}C_{ring}$]DHP after different times of incubation

Incubation (d)	Released as $^{14}CO_2$	% ^{14}C-radioactivity		Calculated degradation of DHP[a]
		Extracted with dioxane	Remainder after dioxane extraction	
0	0	100	0	0
1	0	98.5	1.5	1.5
3	2.1	93.3	4.3	6.4
6	19.6	64.4	14.1	33.7
9	26.5	55.5	14.3	40.8

In order to retard the degradation of [$^{14}C_{ring}$]DHP the number of the bacteria was reduced (A_{600} = 0.5) and incubation was performed in diluted normal medium

[a] Calculation of total degradation of DHP by adding the amount of $^{14}CO_2$ and dioxane-unextractable ^{14}C (biomass)

Quantitative and qualitative aspects of DHP degradation

The intention of the following experiments was to correlate these conclusions further with molecular events occurring in DHP. For this, [$^{14}C_{ring}$]DHP was incubated in the presence and absence of the bacteria. Incubations were terminated at different time intervals and quantitative determinations were performed on the ^{14}C-label released either as $^{14}CO_2$, as soluble in dioxane or as remaining unextractable in the cells (Table 2). Parallel to these, also under quantitative aspects, gel permeation chromatographic analyses were conducted with dioxane extracts from the respective cultures (Fig. 5). It is obvious that (Table 2), depending on the time, part of the ^{14}C-label became unextractable by dioxane, reaching a level of about 14% of the total radioactivity supplied and simultaneously, starting after 3 days, increasing amounts of $^{14}CO_2$ were released.

The chromatographic analyses are illustrated in Fig. 5. The elution profile of the dioxane extract from the incubation of DHP with the bacteria was compared with that from the inoculated control traced by measuring radioactivity. The most striking observation was that a dissociation of the DHP in the uninoculated controls took place producing fractions of DHP with lower molecular weight as time increased. This shifting of DHP to lower molecular fractions was additionally demonstrated by means of high performance chromatography as documented in Fig. 6. The occur-

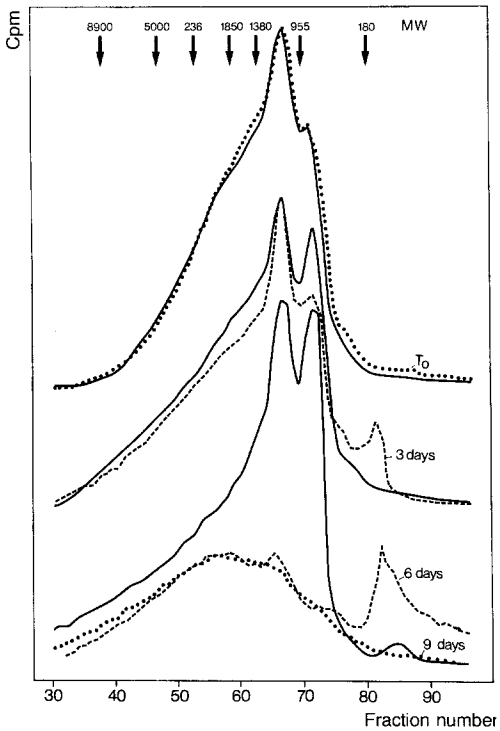

Fig. 5. Gel permeation chromatography of [$^{14}C_{ring}$]DHP after incubation with or without the bacteria at different times: LH-60 column (1.6 cm diam., length 151 cm) eluted with DMF −0.1 M LiCl; distribution of ^{14}C-label in fractions of 2.5 ml each. Styrene standards were used as molecular markers. [^{14}C]DHP from the respective uninoculated control (———) is compared with [^{14}C]DHP from the adequate incubation with the bacteria. Distribution of the ^{14}C-label from controls after 6 d and after 9 d of incubation is identical (not drawn for 9 d incubation). Comparisons were performed quantitatively

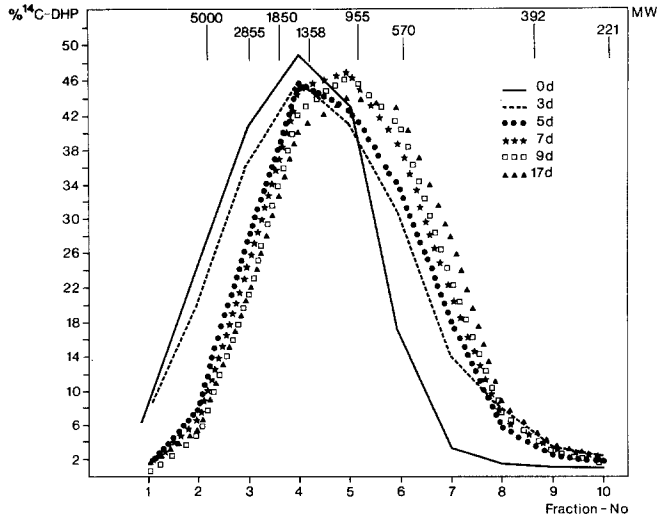

Fig. 6. Dissociation of purified [$^{14}C_{ring}$]DHP in uninoculated medium under experimental conditions. Characterization of the [^{14}C]DHP by gel permeation chromatography (HPLC). Samples were acetylated and fractionated with THF. Radioactivity was measured in the effluents from the columns. Calibration was performed with styrene standards

rence of lower molecular fractions of the DHP could partially explain the stimulation of the bacterial ligninolytic activity described above. However, the dissociation of DHP alone does not explain the fact that higher molecular fractions of the DHP are also degraded by the bacteria. From Fig. 5 it can also be seen that only a small amount of degradation products could be detected in the medium. These compounds are obviously short-lived, they are taken up by the cells as soon as they appear because no accumulation of these substances could be observed.

Incorporation of the ^{14}C-label into the bacterial biomass

As was shown in Table 2, part of the ^{14}C-label employed could not be extracted by dioxane and the question was raised whether this residual radioactivity indicates DHP strongly adsorbed onto the cells or reflects metabolized ^{14}C becoming part of the bacterial biomass. For this, bacteria after the incubation with [^{14}C]DHP for 25 d were exhaustively extracted with dioxane, hydrolyzed with NaOH and fractionated according to the method of Schmidt and Thannhauser (1945). By determination of the radioactivity in the respective fractions an attribution of the ^{14}C-label to different biopolymers was possible. The main incorporation of the label was found to occur with the RNA-fraction (67% of the total radioactivity in extracted cells), followed by 16% incorporation into the protein-fraction, and about 8% of the label was measured in the DNA-fraction. Direct evidence of the incorporation of ^{14}C into RNA could be obtained from the isolation of ribosomal RNA (Kern 1975) from these dioxane-extracted bacteria showing a slight incorporation of ^{14}C at 25 Bq/mg rRNA.

Independent of these chemical analyses, the incorporation of the label into the bacterial biomass could be demonstrated by biological methods, as described under methods. On the basis of appropiate controls, it could be concluded that $^{14}CO_2$ released by *Proteus vulgaris* could only originate from molecules whose radioactivity reflects ^{14}C metabolized and incorporated into the biomass of the ligninolytic bacteria. Depending on the duration of the action of the ligninolytic bacteria upon [^{14}C]DHP, the subsequent incubation of the killed bacteria with *Proteus vulgaris* led to the release of $^{14}CO_2$ which indicated that the incorporation of the label into the biomass of the ligninolytic bacteria reaches a certain level. Calculations showed that for instance after 8 d of incubation with the ligninolytic bacteria about 15% of the applied radioactivity (DHP) was converted to $^{14}CO_2$ and 14.1% of the total ^{14}C-label became insoluble in dioxane. The latter fraction was mineralized by *Proteus vulgaris*. The conversion of incorporated radioactivity to $^{14}CO_2$ occurred at about 30%.

In order to confirm the ligninolytic activity of the bacteria further, lignocellulose complexes, obtained by feeding corn plants *(Zea mays)* with ^{14}C ferulic acid, were also used. The incubations were carried out under the same conditions with equal number of bacteria without or with preincubation with ferulic acid (Table 3). Moreover, a special DHP preparation was included in these tests, which had been polymerized on a matrix and chemically fractionated (Kern, unpublished). As can be seen from the data in Table 3, a slightly higher ligninolytic activity of the bacteria towards theses compounds could be achieved by preincubating the bacteria with ferulic acid. However, the bacteria are also apparently capable of lignin degradation without any experimentally performed stimulation (cf. Figs. 3a, b and 4).

Table 3. Bacterial mineralization of ^{14}C-labeled lignocellulose-complexes (LC) after 21 d

Preparation	Spec. radioactivity (Bq/mg)	kBq/assay	Bacteria without preincubation	Bacteria after preincubation with ferulic acid (2.6 mM)
[a] LC [$O^{14}CH_3$]	27.3	1.365	2.9%	3.5%
LC [^{14}C-β]	10.0	1.000	3.6%	3.5%
LC [^{14}C-γ]	9.6	0.966	4.6%	5.7%
[b] DHP [$^{14}C_{ring}$]-Dioxane-fraction	N.D.	0.958	4.4%	5.3%

[a] Lignocellulose-complexes from corn plants *(Zea mays)* fed with specifically ^{14}C-labeled ferulic acid. Determination of $^{14}CO_2$ in duplicate assays

[b] DHP polymerized on a matrix and, after preextraction with ethanol, extracted from the matrix with dioxane-water (96:4 v/v). N.D. = not determined

Discussion

The studies described were carried out on bacteria whose ligninolytic activity was mainly derived from their ability to oxidize various specifically ^{14}C-labeled DHP preparations. With regard to the fact that DHP's are lignin model compounds and not identical with natural lignin (Nimz and Lüdemann 1976; Brunow and Lundquist 1980) it seemed necessary to perform critical examinations of the DHP-preparations themselves and, in addition to measuring the released $^{14}CO_2$, to get information about the way the DHP is degraded by the bacteria.

Some important parameters in lignin degradation have become apparent from intensive studies on the white rot fungus *Phanerochaete chrysosporium* and have led to the knowledge of the principles of lignin degradation by this fungus (Kirk 1981). However, these principles only have a limited range of validity. Predictions of the ligninolytic organisms in question cannot be made by deduction by any means. Experimental examination of the different parameters still proves to be essential. As a result of systematic investigations some unique properties of the bacteria could be detected as compiled in Table 1. A surprisingly high degradation of DHP by the bacteria was measured within a relatively short incubation period, a fact which is itself of interest. Moreover, the bacteria possess an extraordinary longevity and can withstand long periods of starvation without diminishing their ligninolytic activity towards DHP. Mineralization of DHP takes place even in 0.9% NaCl, moreover over a broad range of pH and at a different pO_2 without remarkable reduction in the release of $^{14}CO_2$. In this context the findings from investigations on *Xanthomonas* sp. (Odier and Monties 1978) that lignin could be degraded anaerobically in the presence of nitrate and glucose were considered but no indication for an anaerobic degradation of DHP could be obtained. These results are in agreement with reports from several authors (Hackett et al. 1977; Zeikus 1980; Zeikus et al. 1982; Odier and Monties 1983).

A critical examination revealed that the DHP preparations used here were not stable. It seemed probable that the water-soluble fractions of DHP and also of dioxane lignin from wheat had a stimulating influence on the bacterial oxidation of the [^{14}C]DHP. This could also be effected by the preincubation of the bacteria in the presence of ferulic acid. Since it is known that this aromate can be utilized by the bacteria as sole carbon source for growth, the pretreatment with ferulic acid causes the synthesis of the enzymes involved in the oxidation of aromatic compounds enabling the cells to mineralize degradation fragments from DHP faster. The conclusion can be made that not necessarily an induction of the ligninolytic activity of the bacteria takes place but rather more the oxidation of degradation products taken up by the cell is enhanced. The dissociation of DHP could still be observed even in the case of DHP preparations which had been further purified in order to remove possibly existent low molecular weight fractions (Fig. 6). Particular attention was paid to this phenomenon and it could be correlated with the bacterial degradation of DHP.

The necessity of critically examining chromatographic separation methods resulted from our own experiences. Various matrices were tested in connection with different elution solvents with respect to their suitability for chromatographic separations of DHP. In agreement with the findings of Connors et al. (1980), bimodal elution profiles were obtained when DMSO, dioxane or DMF were used as eluant. Appropriate re-chromatography demonstrated that this was the reflection of intramolecular association effects and by no means separations according to the respective molecular weight. However, reproducible separations of [^{14}C]DHP, tested by re-chromatography, could be achieved if Sephadex LH-60 was used as the matrix and DMF containing 0.1 M LiCl as the eluant (Connors et al. 1980).

It can be stated that during the incubation of [^{14}C]DHP with the bacteria, degradation of this compound is reflected by the release of $^{14}CO_2$ and by the incorporation of part of the ^{14}C-radioactivity into the biomass of the bacteria. Since degradation of DHP means the conversion of this compound to any form of ^{14}C-labeled compound that is chemically different from the DHP preparation used, calculation of the degradation should base upon the sum of ^{14}C released as $^{14}CO_2$ and that being incorporated into the biomass, as indicated in Table 2. This is a reasonable approach for the quantitative estimation of DHP degradation. It has been shown that during bacterial action upon DHP low amounts of short-lived low molecular weight compounds (Fig. 5) could be detected which do not accumulate. It seems that intermediate structural entities are oxidized to CO_2 by the bacteria as soon as they are formed. We could support this assumption further by analogous experiments using ^{13}C-enriched DHP's (Haider et al., unpublished). The ^{13}C-NMR spectra of these DHP preparations showed after incubation with the bacteria only a few weak new signals which, however, were nearly identical

with those detected during action of a fungus *(Sporotrichum pulverulentum)* on these ^{13}C-enriched DHP's.

Although the results from investigations on the bacterial degradation of DHP were cautiously interpreted, ligninolysis should also be demonstrated using natural lignins. In some cases dioxane lignin from wheat was used which, however, presents an acidolysis lignin and is, with respect to chemical modifications occurring during preparation, only of limited value (Crawford 1981). A clear confirmation of the ligninolytic activity of the bacteria could be obtained by using ^{14}C-labeled lignocellulose complexes (Table 3). In this context it was also shown that special preparations of [^{14}C]DHP were useful for this purpose. Obviously, it is possible to synthesize DHP whose degradation by the bacteria occurs in a similar magnitude as in the case of natural lignocellulose complexes.

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