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Azospirillum Strains Use Phenolic Compounds as Intermediates for Electron Transfer Under Oxygen-Limiting Conditions

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Abstract. The effects of catechol, vanillic, caffeic (CAF), 2-hydroxyphenylacetic, 4-hydroxy- and 3,4-dihydroxybenzoic (3,4-DHBA) acids on the growth of a common rice rhizosphere inhabitant, *Azospirillum lipoferum* were studied. Two strains of this nonfermenting nitrogen-fixing bacterium were used: a motile strain (4B), and a nonmotile strain (4T). Under atmospheric conditions (pO_2 = 21 kPa), the growth of strain 4T was inhibited by catechol (0.1 mM) only. None of these compounds affected the growth of strain 4B. Under 5 kPa O_2, no effect was observed on strain 4B, whereas three of the six tested phenolics stimulated the growth of strain 4T; maximum effects were observed for 3,4-DHBA and CAF. As revealed by TLC and HPLC, under low oxygen, more new lipophilic compounds were formed from CAF by strain 4T, differing from CAF autooxydation products and from the products obtained under 21 kPa O_2. It was hypothesized that strain 4T had the ability to use an oxidized derivative of CAF as a terminal electron acceptor. This hypothesis was tested in experiments under nitrogen-fixing conditions, in the absence of oxygen, and in the presence of N_2O as a reoxidizing agent for CAF. Acetylene was used both as a substrate to measure nitrogenase activity (ARA) and to inhibit the biological transfer of electrons to N_2O. The addition of CAF in the presence of N_2O had the same effect on ARA rates as an addition of oxygen. It is concluded that the strain 4T of *Azospirillum lipoferum* is able to sustain some of its activities (e.g., N_2 fixation) using phenolics as alternative electron acceptors under low oxygen conditions.

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Introduction

Phenolic compounds and their derivatives are present everywhere in the soil environment: phenolics are part of root exudates [42] and intermediates in the biodegradation of molecules containing aromatic rings (lignin, tannins from plant sources, xenobiotics from agricultural sources) [6]. They are found in water extracts of soils, roots and leaf litter [46]. They influence soil biological processes through several interactions. They are involved in chemotaxis [19, 25], allelopathic processes [21], nodulation of plants by symbiotic micoorganisms [5, 25, 33], or plant–microbe recognition processes [35]. They can be used by microorganisms as a source of carbon and energy [4, 18, 26, 27]. A key feature of the microbial degradation of aromatic structures is the ortho-dihydroxylation of the aromatic ring [1, 9, 26]. The resulting polyphenolic compounds, especially 3,4-DHBA (protocatechuic acid), are widely distributed in soils [29]. These polyphenolic compounds may undergo redox reactions involving electron transfers to molecular oxygen, to other organic compounds, including various phenolics [12, 31], or to metals [10, 32]. Moreover, in soils, the polycondensation process of polyphenolic molecules is considered the basic mechanism for the genesis of humic compounds [12, 37, 30] and involves molecular oxygen consumption. Thus, by modifying electron transfers and O_2 concentrations, phenolic compounds could affect microbial activities in soils.

Many activities of soil microorganisms require special O_2 conditions, particularly denitrification and nitrogen fixation. It is well-known that N_2 fixers have low optimal pO_2 for their nitrogenase activity [7]. Heulin et al. [24], for instance, demonstrated that *Azospirillum* nitrogen fixation (acetylene reduction activity) was maximum at O_2 concentrations close to 1%, and ceased with O_2 concentrations reached 8%. Such optimal O_2 conditions are encountered in the root environment of many crops [13, 41]. In these rhizosphere environments, nitrogen-fixing microorganisms also find the root exudates [34] to be an abundant source of carbon. To produce the energy necessary to support their growth and nitrogenase activity [23] when pO_2 is too low, alternative electron acceptors such as NO_3^- are required. The possibility that some phenolics also could play this role, cannot be rejected, due to their above-mentioned chemical properties.

Previous studies have shown some diversity in the metabolism of phenolic compounds by *Azospirillum* spp. It was observed that about 40% of the tested *Azospirillum* strains were able to metabolize benzoate or phenol in the presence of easily utilizable co-subtrates [3]. In that respect, strain 4T of *Azospirillum lipoferum*, isolated from the rhizosphere of rice by Thomas-Bauzon et al. [40], exhibited interesting properties: a laccase activity [17] and the ability to produce melanin-like compounds [16].

All these observations prompted us to undertake a study of the effects of simple phenolic compounds on *Azospirillum lipoferum*, comparing strain 4T with a more typical strain, 4B. A preliminary growth study under various pO_2 levels confirmed that the effect of some phenolics was dependent on the redox status. The interaction between strains of *Azospirillum lipoferum* and phenolic compounds was then investigated more precisely, using nitrogenase activity, measured through acetylene reduction activity (ARA), to monitor the bacterial metabolism with a greater sensitivity than growth measurements. In aerobic nitrogen fixers like *Azospirillum*, ATP is essentially generated by the respiratory electron transport chain. If nitroge-

nase activity is limited by the final electron acceptor concentration, ARA measurements constitute a handy and sensitive indirect measurement of the electron acceptor availability. This was used here to check the hypothesis that phenolic compounds could be used as alternative electron acceptors. In the reported experiments, the compared electron acceptors were O_2 (in low concentrations to avoid ARA inhibition) and a phenolic compound (caffeic acid), alone or in combination with N_2O. Under these conditions, N_2O could not be used as an electron acceptor, as bacterial N_2O reductase was inhibited by acetylene [47]. The role of N_2O was to restore the oxidized form of the phenolic compound whenever the reduced form was produced by electron transfer from bacteria.

Materials and Methods

Phenolic Molecules

Six phenolic compounds with increasing side chain lengths were chosen: catechol (CAT); 4-hydroxybenzoic acid (4HBA); 3,4-dihydroxybenzoic acid (protocatechuic acid, 3,4-DHBA); 3-methoxy-4-hydroxybenzoic acid (vanillic acid, VAN); 2-hydroxyphenylacetic acid (2-HPAA), and 3,4-dihydroxycinnamic acid (caffeic acid, CAF). CAF, VAN, 4-HBA, and 3,4-DHBA are oxidative byproducts of microbial degradation of lignin-derivative compounds (ferulic and coumaric acids) [12]. CAT,3,4-DHBA, and CAF rings carry two hydroxyl groups (in ortho position), whereas 4-HBA, 2-HPAA, and VAN rings carry only one hydroxyl group in ortho or para position to the acidic side chain. On the basis of results from the growth experiments (see below), caffeic acid (CAF) was selected for the electron transfer study.

Bacterial Strains

Azospirillum lipoferum 4B and 4T strains were isolated by Thomas-Bauzon et al. [40] from the rhizosphere of rice, and, more precisely, from the same individual plant. The two strains differ by morphological, genetic, and biochemical characteristics. Strain 4T is a nonmotile rod, possesses a unique laccase activity [17], and aged colonies produce melanin-like compounds [16]. Strain 4B is a regular motile *Azospirillum*, without laccase activity. The two strains have a slightly different plasmid profile [2]. The nature and purity of strains were checked by microscopic examinations and syringaldazide tests according to Givaudan et al. [17].

Both strains were grown in 5 ml Luria broth. Tubes were shaken overnight at 28°C. Bacterial cells were pelleted by centrifugation (15 min, 5000*g*), washed 3 times with sterile 0.7% NaCl, and then resuspended in 2 ml of sterile 0.7% NaCl solution for further assays.

Growth Medium

The effect of various electron acceptor conditions on strain growth was investigated by cultivation of the bacteria in a specific medium, supplemented or not with phenolic compounds. Solution 1: NH_4Cl, 1 g; K_2HPO_4, 3 g; KH_2PO_4, 2 g; malic acid, 5 g; deionized water, 948 ml; pH (adjusted by 1 M KOH), 6.8 Solution 2: $MgSO_4 \cdot 7H_20$, 200 mg; $CaCl_2 \cdot 2H_2O$, 26 mg; NaCl, 100 mg; $Na_2MoO_4 \cdot 2H_2O$, 2 mg; $MnCl_2 \cdot 4H_2O$, 7 mg; deionized water, 50 ml. Solution 3: $FeSO_4 \cdot 7H_2O$, 631 mg; EDTA $\cdot$ $2H_2O$, 529 mg; deionized water, 50 ml. All solutions were autocalved separately at 120°C for 20 min. Solution 1 and solution 2 were mixed and then supplemented with 1 ml of solution 3. One milliliter of a filter-sterilized solution of biotin (0.1 g liter^{-1}) was added in the mixture to obtain a final concentration

of 10^{-5} g liter^{-1}. Filter-sterilized phenolic compound solutions (10 mM) were added when required in growth flasks to a 0.1 mM final concentration.

Growth Conditions

For growth experiments, 0.05 ml of cell suspension in 0.7% NaCl was introduced in 150-ml incubation flasks containing 30 ml of the above growth medium. Two oxygen levels in flask head spaces were used: atmospheric conditions, i.e., around 21% O_2 (O_2 partial pressure = 21 kPa) and 5% O_2 (O_2 partial pressure = 5 kPa). Flasks with atmospheric O_2 were capped with cotton plugs whereas flasks with low pO_2 with capped with gas-tight rubber stoppers. In the low pO_2 treatments, flasks were evacuated 3 times, filled with Helium (high grade) of which 5% was then replaced by pure O_2 before injecting the inoculum and the phenolic compound. Cultivation under atmospheric conditions was performed for 48 h in the dark at 28°C on a horizontal rotary shaker (60 rpm). One-milliliter medium samples were taken after 0, 18, 24, 39, 44, and 48 h of incubation. Cultivation under low O_2 conditions was performed in sealed flasks for 88 h under the same conditions. One milliliter medium samples were taken after 0, 22, 44, 48, 64, 69, and 88 h of incubation. Each incubation was performed in triplicate. Control incubations were conducted without phenolic compounds added. Bacterial growth was measured by optical density of cell suspensions at 540 nm.

Acetylene Reduction Activity (ARA)

Flasks (150 ml) were filled with 30 ml of N-free medium (growth medium described above, without NH_4Cl), evacuated 3 times, and then filled using Helium plus 10% C_2H_2. The residual O_2 partial pressure, measured by gas chromatography (catharometer detector), was less than 0.1 kPa. One-milliliter aliquot volumes of cell suspension in 0.7% NaCl sterile solution were introduced into the flasks. Flasks were incubated at 28°C in the dark, without agitation for 120 h. At this time various treatments were applied: control, 0.3 ml of 0.7% NaCl sterile solution; CAF, 0.3 ml of 10 mM solution of CAF; O_2, O_2 introduced into the flask headspace to a partial pressure of 0.5 kPA; N_2O, N_2O introduced into the flask headspace to a partial pressure of 1.3 kPa; CAF + N_2O, 0.3 ml of 10 mM solution of CAF and N_2O introduced into the flask headspace to a partial pressure of 1.3 kPa. Treatments were carried out in triplicate. Flasks were further incubated without shaking for 204 h (total incubation time was 324 h). ARA was assayed after 0, 18, 22, 36, 66, 88, 100, 120, 144, 188, 200, 220, 280, 310, and 324 h of incubation. Ethylene in flasks was measured by gas chromatography (FID detector).

Redox Potential

The redox potential (Eh) was measured by a platinum electrode (Ingold Pt 4850 DXUS8, AG Industrie, Steinbach, Germany) connected to a mV meter (CG 837, SOLEA, Lyon, France). The electrode was placed in the culture medium immediately after opening the flask. Redox potential values changed from high values on electrode insertion, to lower values when equilibrated, and then to higher values again when atmospheric O_2 diffused into the liquid medium. The values of the lower point of the curve were considered as reflecting the redox potential of the medium.

Caffeic Acid Metabolites Extraction and Characterization

Thin layer (TLC) and high performance liquid (HPLC) chromatography were used to characterize caffeic acid and its metabolites. Metabolites of CAF were extracted from the medium after an 88-h incubation by ethylacetate (vol/vol, 3 times). The extracts were concentrated under vacuum in a rotary

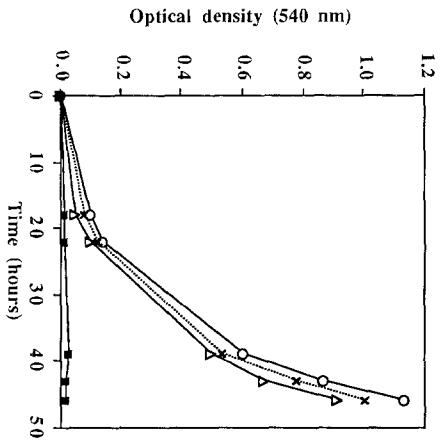

Fig. 1. Influence of the selected phenolic compounds on growth of strain 4T of *Azospirillum lipoferum* under atmospheric conditions. The standard errors of the means were <5% in all cases. Symbols: (—o—), control; (— —), 3.4-DHBA; (—■—), catechol; (···×···), CAF. 4-HBA, 2-HPAA, and VAN (not represented) gave results undistinguishable from CAF, 3,4-DHBA, and control.

evaporator and dissolved in 0.1 ml of high-grade methanol. TLC was performed on aluminum sheets coated with silica gel F254 (Merck, Darmstadt, Germany). CAF and its metabolites were separated by migration in toluene: acetic acid (5:1) mixture. TLC chromatograms were observed at 254 and 360 nm, and compounds were revealed by the Merck diazotized benzidin reagent for phenols. HPLC was performed on a 625L System, equipped with a Microbondapak C18 column (Waters, Milford, Mass.). A first analysis was performed using a gradient of H_2O + 2% acetic acid (A) and acetonitrile (B) (WAA gradient). The WAA gradient conditions were as follows: 0–40 min, A = 100%; 40–45 min, A/B = 60/40; 45–50 min, A = 100%; the flow rate was 1.5 ml min^{-1}. A second analysis was done under isocratic conditions with pure acetonitrile (flow rate 1.5 ml min^{-1}). Eluted compounds were detected using a 991 Photodiode Array Detector (Waters).

Results

Growth

The influence of the chosen phenolic compounds on the growth of strains 4B and 4T of *A. lipoferum* was investigated first. Under atmospheric conditions, growth of strain 4B was not affected by any of the studied compounds (data not shown). The growth of strain 4T was strongly inhibited by CAT, whereas the other compounds (3,4-DHBA, 4-HBA, CAF, VAN, and 2-HPAA) had no influence (Fig. 1). Under 5 kPa O_2, the growth of strain 4T was stimulated by three of the six tested compounds, namely 3,4-DHBA $\geq$ CAF > 2-HPAA (Fig. 2). VAN, CAT, and 4-HBA either slightly stimulated or did not influence growth of strain 4T. Under this low O_2 partial pressure, growth of strain 4B was not influenced by any of these phenolic compounds (data not shown).

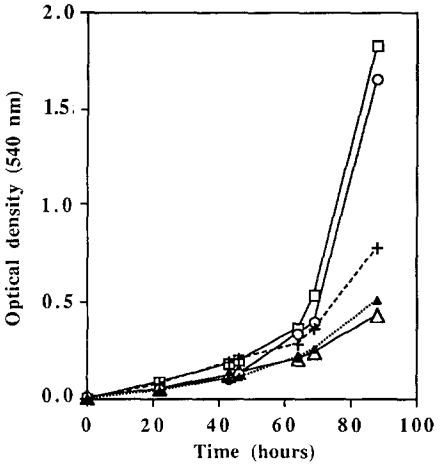

Fig. 2. The growth stimulation of strain 4T of *Azospirillum lipoferum* by phenolics under 5 kPa O_2. The standard errors of the means were <5% in all cases. Symbols: (— —), control; (—□—), 3,4-DHBA; (—o—), CAF; (– – + – –), 2-HPAA; (···▲···), catechol. 4-HBA and VAN (not represented) gave results undistinguishable from CAT and the control.

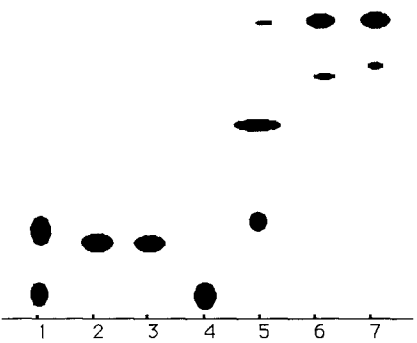

Fig. 3. TLC chromatogram of the products of caffeic acid incubation with *Azospirillum* strains under 21 kPa O_2 and 5 kPa O_2 conditions. Run number: 1, caffeic acid fresh standard solution; 2, caffeic acid + strain 4B under 21 kPa O_2; 3, caffeic acid + strain 4T under 21 kPa O_2; 4, caffeic acid + strain 4B under 5 kPa O_2; 5, caffeic acid + strain 4T under 5 kPa O_2; 6, caffeic acid, sterile incubation under 5 kPa O_2; 7, caffeic acid, sterile incubation under 21 kPa O_2. This figure depicts the compilation of the spot locations as revealed by UV light (254 and 360 nm) and the Merck diazotized benzidin reagent for phenols.

Thin Layer Chromatography

TLC chromatograms are summarized in Fig. 3. The ethylacetate/methanol extract of non-incubated, sterile, fresh caffeic acid solution was eluted as two spots exhibiting bright blue fluorescence. Extracts from caffeic acid incubated (88 h) under sterile conditions in both atmospheric and 5 kPa partial pressure O_2 conditions were eluted as two spots with higher R_f, values showing a much more lipophilic characteristic than components from the fresh caffeic acid solution.

Table 1. HPLC analysis of major products resulting from the incubation of strains 4T and 4B of *Azospirillum lipoferum* with caffeic acid. For HPLC conditions see Text (Materials and Methods)

	WAA gradient			Pure acetonitrile		
Treatment	Peak number	λmax (O.D.)	Retention time (min)	Peak number	λmax (O.D.)	Retention time (min)
CAF (fresh solution)	1	237 (0.18)	16.75	none		
	E_{max}	323 (0.30)				
	2	237 (0.21)	17.80	none		
		323 (0.35)				
CAF (atm. condit.)[a]	none			1	272 (0.18)	1.4
4B + CAF (atm. condit.)	1	234 (0.41)	18.16	none		
		323 (0.64)				
4T + CAF (atm. condit.)	1	237 (0.11)	17.96	none		
		323 (0.18)				
CAF (5 kPa O_2)	none			1	280 (0.24)	3.4
4B + CAF (5 kPa O_2)	1	225 (0.14)	17.35	none		
		345 (0.11)				
	2	234 (traces)	17.96	none		
		327 (traces)				
4T + CAF (5 kPa O_2)	1	236 (0.11)	17.92	1	247 (0.6)	2.12
		323 (0.18)			334 (0.35)	
	2	230 (0.18)	22.82	2	281 (0.1)	2.4
				3	280 (0.1)	3.4

[a]Atm. condit., atmospheric conditions of 21kPa O_2.

Incubation under atmospheric O_2 with both strains resulted in a one-spot chromatogram. This spot exhibited similar R_f but weaker blue/yellow fluorescence when compared to the higher R_f spot of fresh caffeic acid. Under low 5 kPa O_2 conditions, contrasting results were obtained with strains 4B and 4T. Under these limiting O_2 conditions, caffeic acid incubated with 4B strain resulted in one spot with the lowest R_f of all the TLC runs. With 4T strain, three spots were obtained: the less lipophilic compound (lower R_f) had a bright blue fluorescence and was close to the more lipophilic component of fresh caffeic acid, but it did not persist after 24 h in the methanol extract or on the chromatogram run; the compound with the more lipophilic R_f was close to the more lipophilic component from sterile caffeic acid incubation under limited 5 kPa O_2; and a new spot appeared in the intermediate zone of R_f.

High Performance Liquid Chromatography

HPLC confirmed and refined most of the TLC results. Results are summarized in Table 1. (1) HPLC analysis of fresh caffeic acid resulted in two peaks (two spots in TLC) eluted at 16.75 and 17.80 min, respectively. Both compounds had identical UV spectra with two absorption maxima at 237 and 323 nm and correspond to the two stereoisomers of caffeic acid; the two isomers were present in nearly equal

concentrations. No peak was detected with pure acetonitrile. (2) Products of caffeic acid incubation under sterile conditions either under 21 or 5 kPa O_2 were eluted by pure acetonitrile only as one peak after 1.4 min and 3.4 min, respectively, and traces of less lipophilic components were eluted in the WAA gradient. (3) Incubation of caffeic acid with strain 4B under 21 kPa O_2 resulted in one peak eluted by the WAA gradient at 18.16 min (i.e., 36 sec later than the second peak of CAF fresh solution) with O.D. maxima at 234 and 323 nm. This absorption spectrum is very similar to the spectra of the two isomers found in the fresh solution of caffeic acid. The same incubation conditions with strain 4T also resulted in one peak, with the same retention time (17.96 min) but with O.D. at 237 and 323 nm, consistently lower (1/4) than with strain 4 B.

Under low O_2 conditions, strains 4B and 4T gave completely different HPLC patterns of caffeic acid biotransformation. (1) Strain 4B products were represented by only traces of 234 to 327-nm absorbing substance at 17.96 min, and by a main peak at 225 and 345 nm which eluted at 17.35 min, i.e., a weakly lipophilic product (as in TLC run). (2) Strain 4T products were partly eluted in the WAA gradient with two main peaks, one at 17.92 min with O.D. maxima at 236 and 323 nm (i.e., very close to the more lipophilic product from fresh caffeic acid solution), and one at 22.82 min with one maximum absorbance peak at 230 nm. This last peak corresponded to a new component unrelated to any other caffeic acid products (as was the intermediate R_f spot in the TLC assay). Three other products were eluted in pure acetonitrile. The main component, a new product, was recovered after 2.12 min and strongly absorbed at 247 and 334 nm. The third component and the product of sterile caffeic acid incubated in 5 kPa O_2 exhibited similar HPLC properties.

Nitrogenase Activity

Nitrogenase activity was assayed by ARA. Only strain 4T of *A. lipoferum* was considered because of its specific reaction when polyphenolics were added in the growth medium and because ARA of strain 4B was not influenced by the addition of caffeic acid in any combinations (data not shown). As described in Materials and Methods, ARA of 4T strain was first assayed for 120 h in the N-free growth medium. After a lag phase (Fig. 4), ARA increased up to 27 nmol of C_2H_4 produced per hour in all flasks, continued until day 3 and then stopped completely. Up to day 3 (66 h), ARA averaged to 20 nmol of C_2H_4 produced per hour and per flask.

At 120 h, the injection of an electron acceptor in the treated samples or of 0.7% NaCl solution in the control flasks, produced a temporary slight increase in ARA (approximately 2–4 nmol h^{-1} $flask^{-1}$). Forty-four hours after this injection (i.e., 164 h after the experiment began), ARA started again in O_2 and N_2O + CAF treatments and continued during the following 86 h until day 10, and then stopped again. At 300 h, ARA had ceased in all treatments. ARA levels of O_2 and N_2O + CAF treatments were similar and higher than in the control, N_2O, or CAF treatments (Fig. 4). N_2O and CAF treatments exhibited the same ARA as the control.

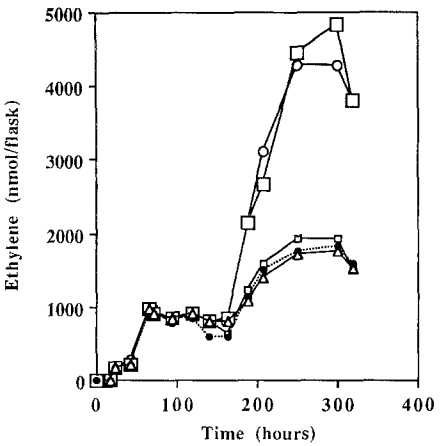

Fig. 4. Acetylene reducing activity of strain 4T of *Azospirillum lipoferum* under electron acceptor-limiting conditions. Caffeic acid and gases were added at hour 120. The standard errors of the means were <4% in all cases. Symbols: (—□—), control; (—○—), treament by O_2; (—□—), treatment by CAF + N_2O; (—●—), treatment by CAF; (—△—), treatment by N_2O.

Redox Potential

During the first 120 h of the ARA experiment, the redox potential decreased from 255 mV to 94 mV (Fig. 5). The introduction of O_2 increased the redox potential of the medium to 120 mV. Redox potential of the samples receiving other electron acceptors was unchanged. In later measurements, the redox potential of control samples was still decreasing progressively. At the same time, the redox potential of N_2O + CAF remained unchanged up to 250 h and then slowly decreased. The difference between N_2O + CAF and control treatments at the end of the experiment was around 20 mV. Redox potential of the medium supplemented by CAF only was slightly higher than the control for 44 h and then rapidly decreased to a lower level than the control. In the O_2 treatment, maximum redox potential was reached by 250 h of incubation (140 mV), and then decreased.

Discussion

The role of phenolic compounds in growth and nitrogenase activity was investigated in two *Azospirillum lipoferum* strains. *A. lipoferum* 4B and 4T were selected for their contrasting properties under high and low oxygen partial pressure and their different interactions with phenolics [16, 17, 24].

The investigated phenolics were chosen as representatives of the various molecular structures of mono- and diphenolic compounds that are likely to be encountered in the rhizosphere environment. Except catechol, each tested compound had a carbon side chain with a carboxylic functional group. With the exception of 2-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, and 3-methoxy-4-hydroxybenzoic acid, studied molecules were diphenols, which are able to form quinoid structures and accept electrons from a convenient electron donor. All the tested molecules

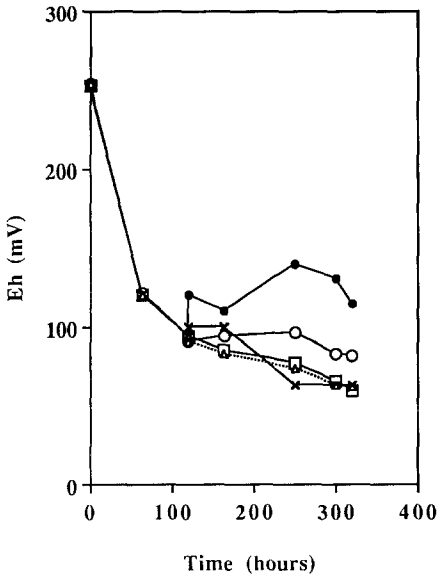

Fig. 5. Redox potential values in the culture medium when strain 4T of *Azospirillum lipoferum* was grown under electron acceptor-limiting conditions. Caffeic acid and gases were added at hour 120. Symbols: (—□—), control; (—●—), treatment by O_2; (—○—), treatment by CAF + N_2O; (—⁎—), treatment by CAF; (···△···), treatment by N_2O.

could also exist in a free radical form with unpaired electrons, a form able to accept electrons as well.

Strain 4B

As far as we know, high oxygen concentrations can depress nitrogenase, but do not limit the growth of *A. lipoferum* strains if the supply of nitrogen is not limiting. In the first experiment, with an ample supply of nitrogen, control treatments under atmospheric oxygen conditions exhibited the highest growth rates. Under these conditions, strain 4B growth was unaffected by the addition of any of the phenolics at 0.1 mM concentrations. The carbon supplied as a phenolic compound was approximately 1% of the carbon provided by malate in the growth medium, so that phenolics could not be expected to have an effect as an extra carbon source. The absence of detectable effects on strain 4B growth means that they are not toxic, whatever the oxygen supply. Nevertheless, TLC and HPLC data showed that caffeic acid was modified by strain 4B. In a fresh solution to CAF, two signals (spots or peaks) were observed, corresponding to its two isomers. In the presence of strain 4B, the incubation of CAF under 21 kPa O_2 resulted in only one compound. The UV spectrum of this compound was very similar to both CAF isomer spectra observed in the fresh solution, with two absorption maxima nearly at the same wavelengths, and a longer retention time. The UV absorption of each of the two peaks of this compound was the sum of the absorption of the corresponding peaks in the UV spectrum of fresh caffeic acid isomers. This strongly suggests that under

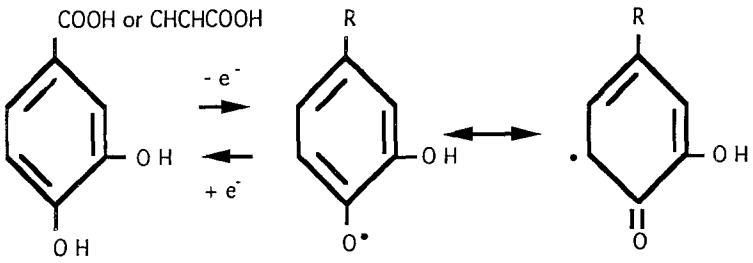

Fig. 6. Possible redox forms and equilibria for caffeic or 3,4-DHBA acid derivatives.

atmospheric conditions, strain 4B did not catabolize CAF but produced a dimer from the two isomers. This unexpected "protection" of the caffeic acid structure against non-biological transformation during the 21 kPa O_2 incubation with strain 4B may be due to the metabolic consumption of reactive components of the medium (dissolved O_2, FeIII) by strain 4B. In this case, it is likely that the bacteria and the molecule had few, if any, interactions. Under low O_2 conditions, only traces of this possible dimer were found together with low amounts of a new phenolic metabolite showing a strong alteration of the CAF molecule.

Strain 4T

Under atmospheric conditions, the addition of a small quantity of phenolic compounds contrastingly affected 4T strain growth. Caffeic acid and 3,4-dihydroxybenzoic acid slightly depressed 4T strain development when catechol dramatically reduced the growth. Catechol reactivity with amino compounds is known [12], as well as the subsequent inhibition of enzymatic activities by orthoquinones [36], but why 4T strain growth was specifically inhibited by catechol remains unclear. Carboxy substituted rings were less active, which could mean that they did not exist directly as quinone but more likely as less oxidized forms, like semiquinone, or with only one unpaired electron as a free radical.

The results of the cultivation of the 4T strain under 5 kPa O_2 showed a strong improvement in growth on 3,4-DHBA and CAF. The contrasting effect of 3,4-DHBA and CAF on strain 4T growth under 5 kPA O_2 (stimulation) and 21 kPa O_2 (very slight depletion) raised the hypothesis that these phenolic compounds could offer an alternative acceptor for electron transfer from bacterial respiration. The electron transfer requires that the phenolic compounds exist, at least partly, under oxidized forms, as shown in Fig. 6. These partially oxidized forms of phenolic acids could be further reduced by electron donors from a chemical process like the FeII/FeIII redox reaction [10, 32], or possibly from bacterial metabolism. The fact that 3,4-DHBA and CAF similarly affected 4T strain growth suggested that the ethylenic group of the side chain of caffeic acid was not involved in the electron transfer process.

The nonmotile 4T strain not only was more sensitive to the presence of the phenolic compounds but also more severely affected the caffeic acid structure in both atmospheric and limited O_2 conditions. Under atmospheric conditions, strain

4T utilized caffeic acid as an additional source of carbon and energy, since HPLC revealed only one-fourth of the initial absorbance of caffeic acid without appearance of other compounds. It is noteworthy that strain 4T under atmospheric conditions and strain 4B under 5 kPa O_2 exerted a similar influence on caffeic acid transformations. Under 5 kPA O_2, the presence of caffeic acid stimulated the growth of the 4T strain whereas four new (compared to the atmospheric incubation) byproducts (Table 1) appeared, one of them exhibiting strong HPLC similarities to one compound produced during the sterile incubation of CAF.

The pattern of byproducts obtained by TLC and HPLC evidenced the complexity of the metabolism of caffeic acid. Under sterile conditions CAF has been transformed by chemical processes. It is well-known that polyphenolics (including CAF) are not stable in aqueous solutions, in contrast to the monophenolic compounds. Transformation by autooxidation [14], photodegradation [39], and interaction with metallic ions [10, 31, 32] have been described. Our TLC and HPLC data showed that similar byproducts of CAF autodegradation (mostly highly lipophilic compounds that were not eluted by the aqueous solutions) were obtained either in atmospheric or in limiting-O_2 conditions (5 kPa). Except for one of the acetonitrile spots of the 4T strain incubation under 5 kPa of O_2, the byproducts of the bacterial metabolism of CAF were different (less lipophilic) from the compounds produced under sterile conditions.

From this first experiment, it could be inferred that the stimulation of 4T strain growth by caffeic acid did not result from the carbon supply but from another process. Under atmospheric conditions this process contributed to the aromatic ring fission and to the assimilation of the resulting aliphatic molecules by the bacteria (a metabolic pathway that was used by the 4B strain under low O_2 conditions only). Under limiting O_2 conditions, this process provided an improvement of electron acceptor accessibility, allowing the bacteria to restore metabolism at the same level as under atmospheric conditions. The question arose whether phenolics would facilitate the access of electrons to remaining traces of oxygen or would constitute an actual alternative electron acceptor after oxygen exhaustion. However, the limitation of O_2 as a metabolic electron acceptor was not easy to demonstrate at 5 kPa O_2 when measuring growth only. It was thus decided to use nitrogenase activity, as *Azospirillum* is a nitrogen-fixing organism. Nitrogenase activity measurement through acetylene reduction (ARA) is a much more sensitive measurement of microbial activity, and it is highly dependent on ATP generated through the respiratory electron transport chain.

In the second experiment, remaining O_2 was rapidly exhausted as an electron acceptor, and the experiment was designed to restore the ATP-generating ability of the respiratory chain with new electron acceptors; oxygen and N_2O. The latter cannot be used directly as the metabolic electron acceptor in the presence of acetylene [45], and its reduction would reveal the role of the phenolic compound as alternative metabolic terminal acceptor. N_2O would generate the oxidized form of the phenolic compound, allowing it to accept new electrons and thus restart ATP generation. This new production of ATP would be immediately revealed by ARA, as under acetylene the use of ATP for growth is very limited [20].

During the first 120 h, strain 4T inoculum consumed most of the electron acceptors present in the medium. The average ARA rate during the active period was slightly lower than expected from the results of a previous study by Heulin et

al. [24]. Twenty nanomoles of C_2H_2 reduced per hour and per 10^8 cells was obtained with a long-term incubation, whereas in short-term incubation (3 h) ARA was undetectable, both under 10 kPA C_2H_2 and 0.5 kPa O_2 [22]. The electron acceptor-limiting conditions were attained when available oxygen had been consumed in the closed vials, i.e., between 36 and 66 h (Fig. 4). At that stage, the addition of oxygen triggered a new phase of ARA, confirming that the amount of electron acceptor was the limiting factor and that enough malate had been supplied as a source of carbon and energy. The introduction of the NaCl solution in the control at 120 h caused a stimulation of ARA after 44 h of further incubation, indicating that some O_2 was introduced during the addition of the reagent solution. The resulting C_2H_4 production (1950 − 911 = 1039 nmol flask^{-1}) corresponded to approximately 2.1×10^{-6} mol of e^- transferred to C_2H_2. The addition of N_2O at 1.3 kPa or of CAF at 0.1 mM concentration resulted in the same low ARA and redox potential changes as in the control treatment. The introduction of O_2 at 0.5 kPa or of N_2O at 1.3 kPa in the flask provided a potential for electron acceptance of approximately 107×10^{-6} mol of e^- and 139×10^{-6} mol of e^-, respectively. As mentioned above, N_2O was not able to directly play the role of electron acceptor for the 4T strain. In contrast, 0.5 kPa O_2 could act as a terminal electron acceptor for strain 4T which exhibited ARA after some hours when O_2 had diffused into the medium and redox potential increased.

For electron transfer, the added CAF at 0.1 mM could accept approximately 3×10^{-6} mol of e^{-1} in the semi-quinone or free radical form, and 6×10^{-6} mol of e^- in the quinone form. CAF introduced in a 95-mV medium should exist in a reduced form only, and, as expected, this treatment did not stimulate ARA. Probably, the low increase of redox potential of the medium immediately after the addition of caffeic acid was due to the existence of a partly oxidized form in the solution at the moment of introduction. The redox potential of the medium supplied with N_2O + CAF remained constant during the later part of the incubation, and the difference in redox values between this treatment and the control was around 20 mV at the end of the experiment. Therefore, the presence of CAF resulted in a 20 mV increase in redox potential (relative to other conditions). This is consistent with the results obtained by Einarsdottir et al. [11] and would suggest the presence of the oxidized form of the phenolic molecule due to the action of N_2O, an oxidizing agent, i.e, a chemical electron acceptor. The cointroduction of CAF and an oxidizing agent resulted in maximal ARA stimulation. In N_2O + CAF treatments, the increase of ARA compared to control was 2893 nmol of C_2H_4 produced. The production of this amount of C_2H_4 required 5.8×10^{-6} mol ($2.9 \times 10^{-6} \times 2$) mol of e^-, which is approximately the e^- transfer capacity of caffeic acid if the quinonic form predominates (6×10^{-6} mol of e^-).

Conclusion

The capacity of phenolic compounds to act chemically as electron donors or acceptors has been recognized for a long time [43, 44], as well as the capacity of electron-transfer properties of phenolics to interfere with the functioning of enzymes [8, 11, 15]. The ability of phenolics to stimulate the respiration of soil microorganisms [38, 45], as well as the enhancement of their nitrogenase activity

[28], has also been reported. However, as far as we know, it has never been suggested that this stimulation proceeds through an enzymatic electron transfer system allowing microaerophilic bacteria, in the absence of oxygen, to use phenolic compounds as intermediates towards a chemical electron acceptor. The above report of the capacity of caffeic acid to be used as an electron transfer molecule by a nonmotile *Azospirillum lipoferum* strain is coincidental with the discovery that such strains also have the capacity to bring about oxidative transformations of phenolic compounds [17]. This suggests that the adaptation of *Azospirillum* to the rice rhizosphere microhabitat could involve the use of phenolic redox couples to refine the tuning of the bacterial environment to a redox value optimal for the functioning of nitrogenase, while keeping the advantage of using the respiratory electron transport chain to meet the high ATP requirement of nitrogen fixation.

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