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# PRODUCTION OF A FERRO-PHENOLIC COMPLEX BY *NOCARDIA OPACA* AND METABOLISM OF PHENOLIC ACIDS IN BACTERIAL CULTURES

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**Key Word Index**—*Nocardia opaca*; bacterium; ferro-phenolic complex; metabolism; *p*-hydroxybenzoic acid; protocatechuic acid.

**Abstract**—During the growth of *Nocardia opaca* on the culture medium containing *p*-hydroxybenzoic acid as a carbon source, protocatechuic acid is generated and quickly becomes a part of the complex with iron and *p*-hydroxybenzoic acid. In the process of a logarithmic phase of the cell's growth, an active hydroxylase of *p*-hydroxybenzoic acid is constantly present in the culture. At the end of this growth phase, the activity of protocatechuate dioxygenase appears as well and the complex undergoes extensive decomposition with its components disappearing gradually from the medium. *p*-Hydroxybenzoic acid, even at high concentration, does not inhibit the growth of bacteria, whereas protocatechuic acid limits it significantly. On the other hand, *p*-hydroxybenzoic acid, which inhibits the activity of protocatechuate dioxygenase, causes excessive accumulation of protocatechuic acid in the culture. Generation of the ferro-phenolic complex which binds these two acids, probably protects the cells from the undesirable influence of their surplus.

## INTRODUCTION

We have shown in our previous papers that certain species of *Nocardia* are able to demethylate veratric, vanillic and isovanillic acids to produce protocatechuic acid formed successively in the medium during cell growth [1, 2]. The production of protocatechuic acid in *Nocardia* may also result from the hydroxylation of *p*-hydroxybenzoic acid [3]. Although protocatechuic acid is toxic to the cells, they are nevertheless able to grow freely, provided that iron ions are present in the medium.

Protocatechuic acid is said to be a terminal phenolic compound in the chain of transformations which lead to disruption of the aromatic ring [4]. In order to convert it finally into aliphatic keto acids (which are further utilized during cell growth), numerous fungi [5] and bacteria [6] as well as *Nocardia* [4] have developed the production of active protocatechuate dioxygenases. This enzyme could successfully detoxify the medium if it were not for the presence of *p*-hydroxybenzoic acid which inhibits its activity. However, it is known that even the presence of highly concentrated phenolic compounds treated as the carbon source does not inhibit free growth of *Nocardia*. It has been shown that under such conditions only a few other bacteria (e.g. *Pseudomonas putida* [7]) are able to grow. This high metabolic adaptation suggests clearly that there must exist additional mechanisms which protect the species from such adverse conditions.

Our work seems to confirm the above suggestion by showing for the first time that the cells of *Nocardia opaca* are protected from the harmful effects of certain phenolic compounds by the production of a ferro-phenolic complex.

## RESULTS AND DISCUSSION

*Nocardia opaca* can grow on a medium which contains *p*-hydroxybenzoic acid as the sole carbon source (Fig. 1).

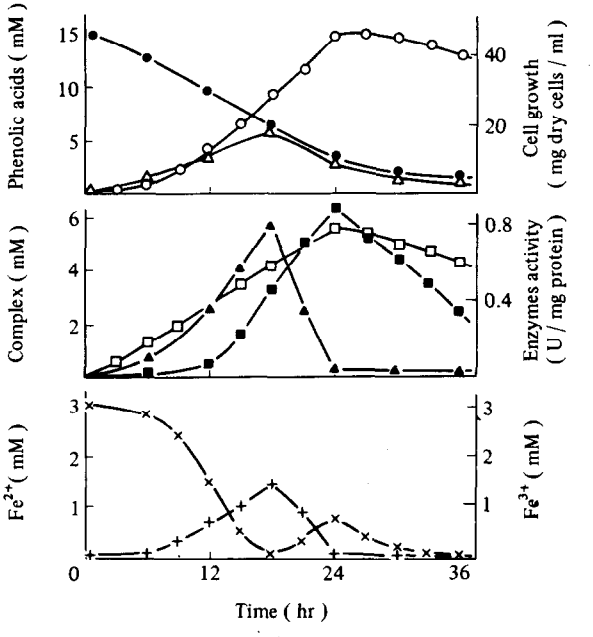


Fig. 1. The course of bacterial growth on the medium containing *p*-hydroxybenzoic acid and iron ions. (○) Cell growth, (●) *p*-hydroxybenzoic acid, (△) protocatechuic acid, (▲) ferro-phenolic complex, (□) *p*-hydroxybenzoic acid hydroxylase activity, (■) protocatechuate dioxygenase activity, (×) ferrous ions (+) ferric ions.

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In the process of culture growth the amount of *p*-hydroxybenzoic acid decreases whereas the level of protocatechuic acid increases (Fig. 1). The process continues until the 18th hr of incubation when the concentration of both acids is identical. From then onwards the concentration decreases until the process of growth is over. At the same time, however, a ferro-phenolic complex is generated and its maximum is reached in the 18th hr of growth. Protocatechuate dioxygenase also becomes active with its peak after 24 hr. The production of cells continues steadily until the plateau is reached after *ca* 25 hr of incubation. Early in the process *p*-hydroxybenzoic acid hydrolase becomes active and after *ca* 12 hr protocatechuate dioxygenase too; their peak occurs after *ca* 24 hr of cell cultivation. An additional amount of protocatechuic acid introduced into the culture inhibits bacterial growth and simultaneously activates protocatechuate dioxygenase (Fig. 2). This enzyme is inhibited by the excess of *p*-hydroxybenzoic acid (Fig. 2). The ferro-phenolic complex was isolated from the culture after 18 hr of incubation (when production was at its highest) and analysed (Table 1). It contains protocatechuic and *p*-hydroxybenzoic acids in equal amounts; iron is bonded to phenolic acids in a molar ratio of 1:5. The complex is stable at pH 7 and above (Table 2). Acidification of the medium causes immediate dissociation and decolorization but basification stabilizes the complex and turns the navy blue and violet colours to a deep red. Qualitative and quantitative composition of the complex is independent of the incubation time of the culture.

Attempts to synthesize the ferro-phenolic complex *in vitro*, i.e. without culture of *Nocardia* were also made. Iron is bound quantitatively by means of protocatechuic acid at pH 8 (Fig. 3). However, under these conditions iron does not react with *p*-hydroxybenzoic acid. The above statements explain the possible occurrence of this compound and iron ions side by side in the first phase of the culture. It is only after the formation of the ferro-protocatechuic complex that *p*-hydroxybenzoic acid is included (Fig. 4). The complex synthesized *in vitro* is identical in composition (Table 1) and behaves at various pHs as the natural complex (Table 2). Moreover, its $M_r$ (640 000) closely corresponds to the molecular weight of the culture complex (650 000). The possible molecular formula of the complex calculated from the analytical data is indicated in Fig. 5.

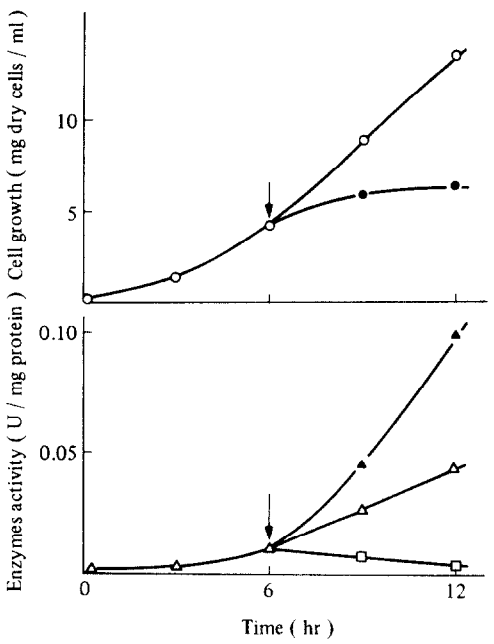


Fig. 2. The effect of phenolic acids on bacterial growth and activity of protocatechuate dioxygenase. (○) Cell growth, (●) cell growth after addition of protocatechuic acid, (△) activity of protocatechuate dioxygenase, (▲) activity of protocatechuate dioxygenase after addition of protocatechuic acid, (□) activity of protocatechuate dioxygenase after addition of *p*-hydroxybenzoic acid. The phenolic acids were introduced into culture at a concentration of 15 mM after six hours of growth (see arrows).

Table 1. Analyses of the ferro-phenolic complexes components

| Methods of analysis* | Components identified (I), components calculated from analytical data (C)† In natural complex | In synthetic complex | Molecular ratio of components Of culture complex | Of synthetic complex |
|---|---|---|---|---|
| TLC | HBA, PCA (I) | HBA, PCA (I) | 1:1 | 1:1 |
| LC | HBA, PCA (I) | HBA, PCA (I) | 1:1 | 1:1 |
| UV | HBA, PCA (I) | HBA, PCA (I) | | |
| IR | HBA, PCA (I) | HBA, PCA (I) | | |
| NMR | HBA, PCA (I) | HBA, PCA (I) | | |
| AAS | Fe (I) | Fe (I) | | |
| EP | HBA, PCA | HBA, PCA | | |
| | $Fe^{3+}$ (I) | $Fe^{3+}$ (I) | 2:2:1 | 2:2:1 |
| MWDTL | HBA, PCA, Fe (C) | HBA, PCA, Fe (C) | 2:2:1 | 2:2:1 |
| MWDGF | HBA, PCA, Fe (C) | HBA, PCA, Fe (C) | 2:2:1 | 2:2:1 |

*LC, liquid chromatography; IR, irradiation spectrophotometry; AAS, Atomic absorption spectrometry; EP, electropolarography; MWDTL, molecular weight determination by TLC; MWDGF, molecular weight determination by gel filtration

†HBA, *p*-Hydroxybenzoic acid; PCA, protocatechuic acid.

Table 2. The dependence on pH of the spectral data of the ferro-phenolic complexes isolated from the culture and synthesized *in vitro*

| | Light absorption (nm) | | | |
|---|---|---|---|---|
| | Natural complex | | Synthetic complex | |
| pH | Maximum | Minimum | Maximum | Minimum |
| 7.0 | 210, 250, 290, 568 | 230, 278, 450 | 210, 248, 290, 568 | 227, 278, 450 |
| 9.0 | 210, 250, 290, 580 | 237, 278, 400 | 212, 250, 290, 580 | 233, 278, 400 |

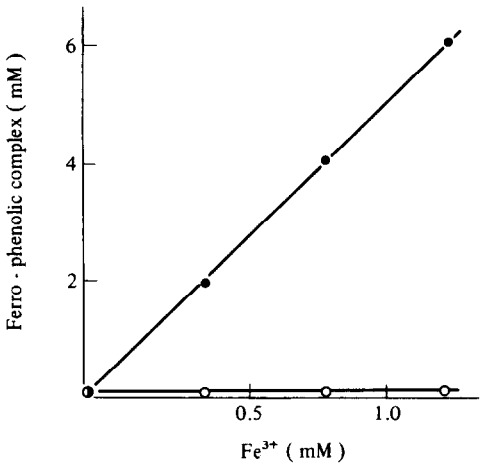


Fig. 3. The *in vitro* synthesis of the ferro-phenolic complexes with ferric ions (ferric chloride) and *p*-hydroxybenzoic acid (○) or protocatechuic acid (●). 6 mM phenolic acids were used in the reaction mixture.

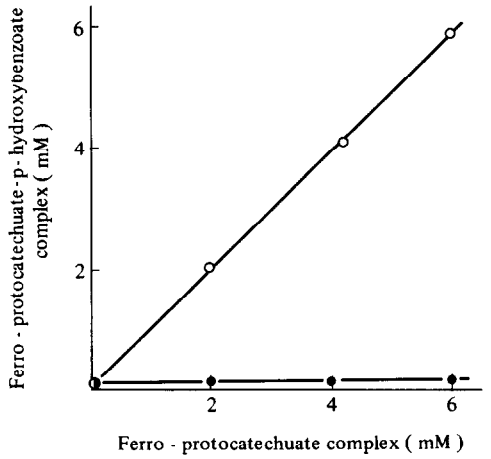


Fig. 4. The *in vitro* synthesis of the ferro-protocatechuate-hydrobenzoate complex with the ferro-protocatechuate complex and *p*-hydroxybenzoic acid (○) or protocatechuic acid (●) 0.6 mM phenolic acids in the reaction mixture were used.

The biochemical processes in the growing cultures either poor or rich in iron, are probably as follows: *p*-hydroxybenzoic acid, though useful as a source of carbon at the very beginning of growth, inhibits activity of

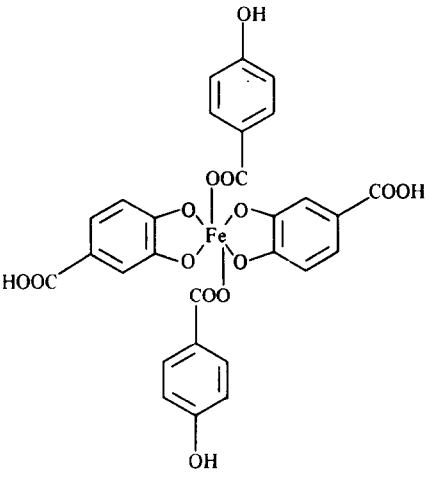


Fig. 5. A molecular formula for the ferro-phenolic complex.

protocatechuate dioxygenase which is being formed at the time of protocatechuic acid appearance. Therefore it prevents the transformation of protocatechuic acid into metabolically useful organic acids. Protocatechuic acid accumulating in the culture affects, in turn, the cell growth. In the second case (i.e. in the presence of iron in the medium) these negative processes are counteracted by iron ions, which first chelate the surplus of protocatechuic acid and then produce a complex with the excess of *p*-hydroxybenzoic acid (the inverse order is not possible). Dioxygenase freed in this way, continuously catalyses ketoacid formation from protocatechuate, which may lower the medium pH and thus cause the complex to dissociate. Later, because of ketoacid consumption by growing bacteria, the pH may increase giving rise to complex formation again. This process is most probably repeated periodically until *p*-hydroxybenzoic acid is used up in the medium.

Iron would then function as an environmental factor preventing the appearance of a toxic surplus of phenolic acids, while the complex resulting from the reaction of phenols and iron would reduce the excessive amounts of free iron ions. This last feature is exhibited also by natural siderophores. They contain orthodiphenolic groups of chelating functionalities which complex heavy metals [8]. However, their origin seems to be connected with amino-acid metabolism [9]. On the other hand some *Nocardia* strains utilize iron ions in a different way. They produce so-called 'nocardiamine' which belongs to the ferroxamine group [10]. This substance functions most probably as a carrier of iron ions and at the same time shows the

characteristics of an antibiotic which protects bacteria from the intrusion of microorganisms from outside. The complex described in this work would thus supplement surplus iron ion distribution or simply replace it in case of highly concentrated phenolic compounds.

Because intrinsic decomposition of the complex begins at the termination of the logarithmic phase of growth, i.e. at the same time the cells start aging, it is necessary to consider the possibility of changes in the permeability of their cell membranes. These changes probably enable *Nocardia* to bind iron directly. In fact we have shown that the complex decomposition and the disappearance of iron ions (including the sum of $Fe^{2+}$ and $Fe^{3+}$) from the medium occur at the same time (Fig. 1).

In the light of the above results, the production of a ferro-phenolic complex in the culture of *Nocardia opaca* and its metabolic function seems logical and easy to understand. It probably functions in the culture as a 'temporary stock' of the substances which are useful for bacteria. These substances, however, if they occur in excessive amounts, disturb bacterial functions. This superfluous amount is therefore neutralized in the process of complex formation.

We have shown in our previous work that protocatechuic acid appears in the culture of *Nocardia opaca* as result of the demethylation of metoxyphenolic acids [1]. Now can be stated that this acid can also be formed, even in large quantities, by hydroxylation of *p*-hydroxybenzoic acid, provided that its toxicity can be neutralized by the formation of a ferro-phenolic complex in the culture.

## EXPERIMENTAL

*Culture conditions.* The strain *Nocardia opaca* (DSM 43202) came from the Collection of Microorganisms at Göttingen, F.R.G. The strain was kept on 2% agar slants as described earlier [1, 11]. *p*-Hydroxybenzoic acid (Fluka, Buchs, Switzerland) at the final concn of 15 mM was dissolved in 100 $\mu$l of 10% KOH and added as the sole C source to the culture contained in 1000 ml medium of 0.4 g of $KH_2PO_4$, 1.6 g of $NH_4Cl$ and 0.2 g of $MgSO_4$. In the case of iron containing cultures, the final concn of $FeSO_4$ was 6 mM and 2 mg of ascorbic acid were added to 1000 ml of the medium.

*Determination of phenolic acid.* The total amount of phenolic compounds in the culture or in the eluates from the columns was determined colorimetrically with diazosulphanilamide (DASA) according to ref. [1]. The concn of *p*-hydroxybenzoic and protocatechuic acids (which occur together as well as separately) was measured by HPLC using a Liquochrom Model 2010 Chromatograph (Budapest, Hungary), with a Chromsil column 25 cm long and 4.6 cm in diameter on which 20 $\mu$l of specimen was applied. The chromatogram was developed using $C_6H_6$–propionic acid –MeOH (22:1:2) and a rate of 2 ml/min. The characterization of phenolic acids in complexes produced both *in vivo* and *in vitro* was based on a $^1H$ NMR spectrum at 80 MHz using $D_2O$ as solvent. The analysis of IR spectrum was done on at 400–4000 $cm^{-1}$ range in KBr.

*Determination of iron.* In order to determine total iron and $Fe^{2+}$ or $Fe^{3+}$ respectively in the post-culture liquid when the cells have been centrifuged 'Aquamerc' test was applied (Merck, Darmstad, F.R.G.) using $A = 522$ nm. The contents of iron in the ferro-phenolic complex was determined by electron impact mass spectrum analysis with Atomic Absorption Spectrometer AAS-3 (Karl Zeiss). Iron oxidation rate in the complex was determined polarographically by means of a mercury-dropping electrode with oscilloscopic recording of the curves and digital reading of the peak heights. The rate of cyclic polarization changes was 20 $vs^{-1}$. The electrode system was DME-SCE-mercury pool [12].

*Determination of enzyme activity.* The activities of *p*-hydroxybenzoic acid hydroxylase and protocatechuate dioxygenase were assayed by polarographic oxygen determination using the Clark oxygen electrode as described in a previous paper [2]. The assay system contained (in 3 ml of 0.01 M phosphate buffer pH 7) 1 $\mu$mol of substrate and 0.2 ml of *Nocardia* cells crude extract prepared according to Engelhardt *et al.* [13]. One unit of specific activity was defined as the amount of enzyme oxidizing 1 $\mu$mol of substrate per minute per mg of protein at 30°.

*Isolation of the ferro-phenolic complex from the culture medium.* The complex was isolated after 18 hr of the culture growth. *Nocardia* cells were centrifuged (10 min at 10000 *g*) and the supernatant was lyophilized. The separation of complex was achieved with Sephadex G-25 column (3 × 50 cm) for 200 mg of lyophilisate dissolved in 2 ml 0.005 M Pi buffer pH 7. The same buffer was used for elution. The complex was visible during its migration on the column as a deep violet band. The complex-rich fractions were kept in eluting buffer or lyophilised.

In vitro *synthesis of a ferro-phenolic complex.* The complex was produced when identical volumes of *p*-hydroxybenzoic (6 mM) and protocatechuic (6 mM) acids were mixed in a vessel containing a magnetic stirrer in the presence of 0.005 M Pi buffer at pH 7. While the mixture was being stirred $FeCl_3$ was added (to the concn of 3 mM), until the colour was fully satd at $A = 568$ nM. The complex was further filtered through Sephadex and kept in eluting buffer or liophilized.

*Determination of $M_r$ of the complexes.* They were determined both by TL-G-25 superfine Sephadex chromatography [14] and by conventional Sephadex G-50 (1 × 100 cm) chromatography.

*Quantitative determination of the complexes.* The culture complex was determined in the post culture liquid when the cells were centrifuged by measuring the extinction at $A = 568$ nm. The concentration of the complex was established on the basis of a calibration curve drawn for various amounts of the *in vitro* synthesized complex.

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