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Inhibition of Imidazoleglycerolphosphate Dehydratase of *Phytophthora Parasitica* by Aminotriazole *in situ* and after Cloning and Expression of the Respective Gene (*HIS3*) in *Escherichia coli*

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Abstract

The *HIS3* gene of *Phytophthora parasitica* was isolated from a plasmid library by complementing *E. coli his*B463 to histidine prototrophy. The gene, encoding imidazoleglycerolphosphate dehydratase, was expressed in *E. coli* independently of the promoter of the cloning vector. The growth of *Phytophthora parasitica* was far more sensitive to 3-amino-1,2,4-triazole (amitrole) than that of the *E. coli* w.t. The inhibition was fully counteracted by histidine. *E. coli* with a defective imidazoleglycerolphosphate dehydratase complemented by the *Phytophthora* gene exhibited the same high sensitivity against amitrole as *Phytophthora* itself. The expression of genes in *E. coli* is suggested for screening potential inhibitors of enzymes in pathogenic organisms.

Zusammenfassung

Inhibition der Imidazolglycerolphosphatdehydratase von *Phytophthora parasitica* durch Aminotriazol *in situ* sowie nach der Klonierung und Expression des jeweiligen Gens (*HIS3*) in *Escherichia coli*

Das *HIS3*-Gen von *Phytophthora parasitica* wurde aus einer Plasmidlibrary isoliert, indem *Escherichia coli his*B463 zur Histidinprototrophie komplementiert wurde. Das Gen, welches das Enzym Imidazolglycerolphosphatdehydratase kodiert, wurde in *E. coli* unabhängig vom Promoter des klonierenden Vektors exprimiert. Das Wachstum von *P. parasitica* war gegen 3-Amino-1,2,4-Triazol (Amitrol) deutlich empfindlicher als das vom *E. coli*-Wildtyp. Die Inhibition wurde durch Histidin vollständig aufgehoben. *E. coli* mit einer defekten Imidazolglycerolphosphatdehydratase aber mit dem *Phytophthora*-Gen komplimentiert, zeigte die gleiche hohe Amitrolsensitivität als *Phytophthora* selbst. Die Expression von Genen in *E. coli* wird als eine Screeningmöglichkeit für potentielle Enzyminhibitoren in pathogenen Organismen vorgeschlagen.

Amitrole (3-amino-1,2,4-triazole) was used extensively world-wide as a herbicide in the past, and in some countries until recently (LOHS and MARTINETZ 1991). New interest in the substance has recently emerged from its potential as a tool in studies in macrophage

biochemistry (BUCHMUELLER-ROUILLER *et al.* 1992) and in the investigation of the regulation of DNA superhelicity in bacteria (TOONE *et al.* 1992). The herbicid effect of amitrole was originally believed to be based on an interference with porphyrin and chlorophyll synthesis, with cation metabolism, and with the synthesis of purines (HILTON *et al.* 1963). Later work, however, defined imidazoleglycerolphosphate dehydratase (IGPD) from the histidine biosynthesis pathway as the only target of amitrole in some fungi and bacteria (HILTON *et al.* 1965, KLOPOTOWSKI and WIATER 1965).

The cloning of genes encoding potential targets for synthetic inhibitors is a prerequisite for their overproduction. This makes it possible to rationally design second generation fungicides, herbicides and other means of chemical crop protection. The most straightforward approach consists of the (i) crystallization of a purified enzyme; (ii) determination of its 3D-structure by X-ray diffraction analysis, and (iii) computer-aided design of an inhibitor molecule which inactivates the enzyme by a specific interaction with an essential part of its structure. This approach will play a key role in inhibitor design in the future, both in plant pathology and human medicine. High costs for the required technology currently make rational inhibitor design available to only a few research groups.

Phytophthora parasitica is a representative of oomycetous protoctists unrelated to fungi, the response of which to amitrole was not yet known. In this work, it is shown that imidazoleglycerolphosphate dehydratase (IGPD) from *P. parasitica* is very sensitive to amitrole and that this sensitivity can also be demonstrated after expressing its gene in *E. coli*. A new approach is suggested for screening of inhibitors of universal metabolic pathways by monitoring the growth of *E. coli* auxotrophic mutants complemented by genes cloned from a target organism.

Material and Methods

Cultivation of *Phytophthora parasitica*

P. parasitica strain DSM-1829 (mating type A2) was grown with gentle shaking at 25 °C in synthetic medium GM7. Preparation of the medium: 3 g L-asparagine, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 50 mg $CaCl_2$, 10 mg $FeCl_3 \cdot 7H_2O$ and 1 mg thiamine were dissolved in distilled water, the pH was adjusted to 5.5 with 2 M NaOH, and the solution was made to 900 ml and autoclaved for 15 min at 120 °C; medium for pouring plates was solidified with 20 g agar per liter. After autoclaving, 100 ml of sterile-filtered glucose solution (200 g/l) was added.

Isolation of *Phytophthora parasitica* DNA

Mycelium was harvested by filtration with a nylon mesh, frozen at − 70 °C, and lyophilized. Total *Phytophthora* DNA was isolated using a modification of the CTAB method (KLIMCZAK and PRELL 1984) and separated into fractions differing in GC-content in CsCl/bisbenzimide gradients according to GARBER and YODER (1983) using a low dye concentration (KARLOVSKY and DECOCK 1991). Nuclear DNA was collected separately from mitochondrial DNA, extracted five times with 3-methyl-1-butanol and precipitated with ethanol.

Construction of the library

P. parasitica nuclear DNA was partially digested with the restriction enzyme *Sau*3A and separated by centrifugation through a 10–40 % sucrose density gradient. Fragments ranging from 7 to 12 kb were collected and cloned into the *Bam*HI site of pUC19 (YANISCH-PERRON *et al.* 1985). Recombinant plasmids were transformed into *E. coli* JM109.

Assay of the inhibitory effect of amitrole

Amitrole (3-amino-1H-1,2,4-triazole) was purchased from Serva. Solid minimal media were supplemented with amitrole from a 0.25 M stock solution in water. GM7 medium was used for *Phytophthora* (see Cultivation of *Phytophthora parasitica*), M9 medium for *E. coli* (MANIATIS *et al.* 1982).

Results and Discussion

Isolation of HIS3 gene of *Phytophthora parasitica*

The library consisting of 52 000 colonies was divided into eight pools which were separately amplified in *E. coli* JM109 (YANISCH-PERRON *et al.* 1985). The analysis of a random sample revealed that 40 % of the plasmids contained inserts with the average size of 7.9 kb. Taking the genome size of *P. parasitica* to be 2.3×10^7 bp (E. MÜHLBAUER, unpublished), the library comprised seven genomic equivalents. The probability for a randomly selected small single copy gene to be cloned was over 0.99. For complementation, 1 to 5×10^9 *E. coli* SB3930 cells carrying the *his*B463 mutation were made competent according to Kushner's protocol (MANIATIS *et al.* 1982) and transformed with each of the eight plasmid pools from the library to produce 1 to 8×10^5 ampicillin resistant colonies. Selection for prototrophy was performed by plating transformants on M9 plates (MANIATIS *et al.* 1982) with ampicillin 30 µg/ml at 35 °C. Twenty-eight His^+ colonies were recovered from the transformation with one plasmid pool; why no complementing plasmid was found in any of the other seven pools is unknown. All His^+ colonies contained the same plasmid with an insert of about 7 kb. One of the plasmids was designated pHS1 and used for further analysis.

Transcription control of *HIS3* in *E. coli*

DNA fragments cloned in the *Bam*HI site of pUC19 are under the control of the *lac* promoter. Strain SB3930 carried a wild type *lac* operon. In such strains, the amount of the *lac* repressor produced is not sufficient to saturate operators on a multicopy plasmid and the *lac* promoter of pUC19 remains derepressed.

In order to test whether the complementing activity of pHS1 depended on the promoter of the vector, a second, compatible plasmid carrying the $lacI^Q$ allele which overexpresses the *lac* repressor, was introduced into transformed cells. The plasmid designated pAC-lacI was constructed by cloning the 1.2 kb *Hin*dIII fragment carrying the $lacI^Q$ gene from pMMB22 (BAGDASARIAN *et al.* 1983) into the *Hin*dIII site in pACYC184 (CHANG and COHEN 1978). *E. coli* SB3930 (pHS1) was transformed with pAC-lacI, and transformants were selected on plates with ampicillin and chloramphenicol.

Expression of the chromosomal *lac*Z gene in double-transformants was followed on plates with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), which is an artificial substrate for β-galactosidase (product of the *lac*Z gene), as a control for the repression of *lac* promoters (Table 1). Expression of *HIS3* was monitored as growth on a medium without histidine. Transformants grew equally well on minimal media with and without the inducer, isopropyl-β-D-thiogalactopyranoside (IPTG), indicating that the gene complementing the *his*B463 mutation was expressed constitutively. Colonies carrying both plasmids were white on plates without IPTG (Table 1). This proved that the amount of the repressor produced by the overexpressing $lacI^Q$ allele on the multicopy plasmid pAC-lacI was sufficient to titrate *lac* operators present in the cell and that the expression of *HIS3* was independent of the *lac* promoter of the vector. It is concluded that a part of the *Phytophthora parasitica* DNA sequence in pHS1 located in front of the *HIS3* gene simulated *E. coli* signals

Table 1
Influence of repression of the *lac* promoter on complementation of *his*B463 by cloned *Phytophthora* DNA

Plasmids	LB + X-gal	LB + X-gal + IPTG	Growth on M9 min medium
No plasmid	white	blue	no
pHS1	blue	blue	yes
pHS1 + pAC-lacI	white	blue	yes

Single colonies of *E. coli* SB3930 (*his*B463, Lac$^+$) carrying plasmids as indicated were picked and transferred to solid media. After 1 day of incubation at 37 °C, growth and colour were examined.

for the initiation of transcription and translation, and controlled the expression of the cloned gene. The same situation was found with the *TRP1* gene of *P. parasitica* (KARLOVSKY and PRELL 1991).

Inhibition of IGPS of *P. parasitica* by amitrole *in situ* and in *E. coli*

The results are summarized in Table 2. Since the growth of *P. parasitica* on GM7 medium was totally inhibited by 2 mM amitrole and since the inhibition was completely released by histidine even at an eight-times higher concentration of amitrole, it was assumed that the only target for amitrole in *P. parasitica* was histidine biosynthesis. Wild-type *E. coli* did not show any sign of inhibition in the concentration range tested. The *his*B463 strain complemented with the cloned *HIS3* gene, however, exhibited the same sensitivity

Table 2
Effect of amitrole on growth of *Phytophthora parasitica* and *E. coli* carrying cloned *HIS3* gene on minimal media

Amitrole (mM)	Histidine (mM)	*E. coli* w.t.	*E. coli his*B with pHS1	*Phytophthora parasitica*
0	0	+++	+++	+++
0.5	0	+++	+++	++
1	0	+++	++	+
2	0	+++	+	–
3	0	+++	–	–
4	0	+++	–	–
8	0	+++	–	–
16	0	+++	–	–
16	0.1	+++	+	+++

Ten μL of overnight cultures of *E. coli* (grown with ampicillin when harbouring pHS1) were spotted on minimal plates containing different concentrations of amitrole; the growth was examined after 3 days at 37 °C. Discs of 5 mm diameter cut out from minimal plates overgrown with mycelium of *P. parasitica* were placed in the center of plates containing amitrole in different concentrations; the plates were incubated at 24 °C and the growth was examined after 8 days.

to amitrole as *P. parasitica*. At a high amitrole concentration, the growth inhibition could be released only partially by externally added histidine. Since the w.t. strain was not inhibited, an interference of amitrole with histidine transport may be the cause of the effect rather than a second target for amitrole in *E. coli*.

The observation that a cloned eukaryotic gene confers sensitivity to a specific inhibitor in *E. coli* presents the possibility to screen potential inhibitors of enzymes from pathogens by using auxotrophic *E. coli* strains complemented by cloned genes encoding these enzymes. As compared to direct tests on pathogens, this approach would significantly speed up the screening in cases where cloned genes encoding target enzymes are available, especially for pathogens that cannot be grown in axenic cultures. The method also has an advantage as compared with biochemical *in vitro* assays based on isolated enzymes. It would be useful in cases where unstable metabolic intermediates have to be enzymatically produced in the assay system as a substrate for the enzyme and in cases where the products are difficult to monitor *in vitro*.

Tested substances may interact in *E. coli* with targets other than the product of the cloned gene. Consequently, the method should be used for comparison of related derivatives rather than for empirical screening of randomly selected compounds. The result will be affected by transport and inactivation of tested substances, so the method should be regarded as the first screen only. On the other hand, general characteristics of a living test system offer extra advantages when screening for biological activity as compared to *in vitro* systems. For example, substances unable to cross the phospholipid membrane of *E. coli* will probably not enter cells of the target pathogen and will be inactive *in vivo* though they may score well in an *in vitro* system.

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