

# Kinetic properties of pure overproduced *Bacillus subtilis* phenylalanyl-tRNA synthetase do not favour its in vivo inhibition by ochratoxin A

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Received 30 April 1993

Ochratoxine A (OTA) inhibits growth of *Bacillus subtilis* at pHs below 7. Since OTA is a phenylalanine analogue, this effect could be due to inhibition of phenylalanine-tRNA synthetase (PheRS) by competition of this mycotoxin with the amino acid. Homogeneous PheRS was purified from *Bacillus subtilis* and from *E. coli* transformed with the PheRS gene. The latter produced about 40 times more PheRS than *B. subtilis*. The  $K_m$  and  $K_i$  values of PheRS, respectively, for phenylalanine and OTA were measured and their concentrations within the cell determined. It appears that the concentration of OTA in the cell, in spite of a 25-fold accumulation, remained too low to significantly compete with phenylalanine. This does not suggest PheRS to be the target of OTA in cell growth and protein synthesis inhibition in *Bacillus subtilis*. It was also shown that the 2–3-fold increase of PheRS in OTA-treated cells is not due to phenylalanine-controlled attenuation regulation.

Ochratoxin A; Phenylalanyl-tRNA synthetase; *Bacillus subtilis*; Growth inhibition

## 1. INTRODUCTION

Ochratoxine A (OTA) is a naturally occurring mycotoxin of the fungal genera *Aspergillus* and *Penicillium* [1]. It is a major causal determinant of porcine nephropathy [2] and has been suggested as a possible disease determinant of Balkan endemic nephropathy which is also closely associated with a high frequency of carcinoma of the renal pelvis, ureter and urinary bladder [3]. Experimentally, OTA has been shown to be nephrotoxic, genotoxic, teratogenic and immunosuppressive [4]. Ochratoxin A also has antibiotic properties. It inhibits growth of Gram-positive bacteria at pH levels lower than 7, but does not affect Gram-negative bacteria [5,6]. If OTA is added to *Bacillus subtilis* cultures during lag phase, this phase is prolonged. After termination of lag phase, the growth rate in the presence of OTA is almost the same as in untreated control cultures and is no longer sensitive to the same concentration ranges of OTA. Prolongation of lag phase is linearly dependent on the concentration of OTA [6]. On the other hand, growth rates are not affected at all by different concentrations of OTA when the mycotoxin is added to the growth medium together with the inoculum [7]. When the concentration of the mycotoxin added to growing cells is higher than about 40  $\mu\text{M}$ , OTA induces autolysis at pH levels lower than 6 [8]. At these concentrations, OTA induces an uncoupling of the respiratory chain [8]. At lower concentration (10  $\mu\text{g/ml}$ )

the uncoupling effect is too small to account for growth inhibition. Nevertheless, at this concentration, OTA completely inhibits protein synthesis after 160 min. At the same time the regulatory nucleotides ppGpp and pppGpp increase about 3–4-fold [9]. The accumulation of these nucleotides is generally interpreted as a sign that some tRNA is incompletely aminoacylated in the cell [10]. Since OTA is a phenylalanine analog, it was possible that the cell starvation effect could be attributed to competitive inhibition of phenylalanyl-tRNA synthetase (PheRS) by this mycotoxin. In fact, Konrad and Rösenthaller [11] showed that OTA inhibits PheRS activity using partially purified *Bacillus subtilis* enzyme. We have tested this hypothesis using pure *Bacillus subtilis* PheRS purified from an *E. coli* strain which overproduces PheRS.

## 2. MATERIALS AND METHODS

### 2.1. Bacterial strains and growth media

*Bacillus subtilis* 168, *E. coli* JM 109, rec A1, endA1, gyrA96, thi, hsdR17, supE44, relA1,  $\lambda^-$ ,  $\Delta(\text{lac-proAB})$  were used [12].

*B. subtilis* 168 was grown in full medium: bacto-peptone 150 g, yeast extract 400 g, Na-glutamate 10 g,  $\text{KH}_2\text{PO}_4 \cdot 3\text{H}_2\text{O}$  140 g,  $\text{KH}_2\text{PO}_4$  60 g, Na-citrate 1 g,  $(\text{NH}_4)_2\text{SO}_4$  2 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  2 g,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , glucose 50 g per 10 l.

*E. coli* JM 109 transformed with the Phe S/T gene was grown in 1 l of LB medium: 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1 mM NaOH. Pure *E. coli* tRNA<sup>Phe</sup> was purchased from Boehringer (Mannheim).

### 2.2. Construction of a PheRS overproducing strain

The thermosensitive *E. coli* PheRS mutant IBPC 1651 [13] was complemented with a *B. subtilis* DNA library.

A 6.6 kbp DNA fragment which complements the thermosensitive defect and carries the pheS/T gene of *B. subtilis*, was inserted in the

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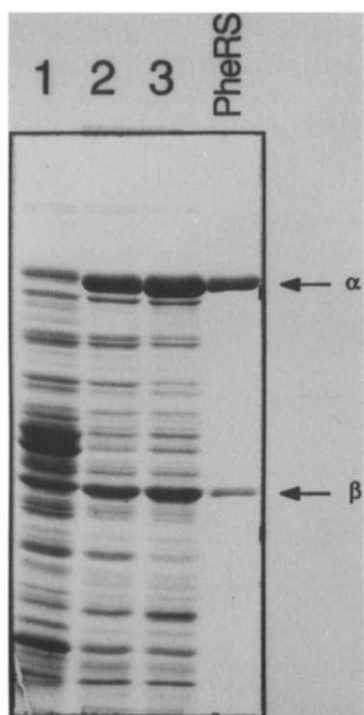


Fig. 1. SDS-polyacrylamide gel electrophoresis of crude extracts from *E. coli* JM 109-bluescript M13 (1), from *E. coli* JM 109-bluescript-M13-*pheS/T* (2 and 3) of pure PheRS. The proteins corresponding to the  $\alpha$  and  $\beta$  subunits are indicated by arrows.

*Pst*I site of bluescript M13 (Stratagene, Paris) and was transformed into *E. coli* JM109.

### 2.3. Preparation of purified phenylalanyl-tRNA synthetase

200 g of *B. subtilis* 168 cells (wet weight), harvested from 14 l of full medium, were washed in buffer A (20 mM Tris-HCl, pH 7.8, 1 mM  $MgCl_2$ , 0.1 mM EDTA, 1 mM mercaptoethanol, 10% glycerol) and sonicated in the same buffer. The crude extract was centrifuged at  $100,000 \times g$  and dialysed against buffer A. A DEAE-cellulose column (300 ml) was charged with the extract and protein eluted with 1,000 ml buffer A and a 0–400 mM KCl gradient. The fractions with PheRS activity were pooled and dialysed against buffer B (Buffer B is the same as buffer A except 100 mM potassium phosphate, pH 7.5, replaces the Tris-HCl). A second DEAE-cellulose column (80 ml) equilibrated in buffer B was charged with the dialysed fractions and protein eluted with 500 ml of buffer B by linearly increasing the potassium phosphate concentration to 350 mM. The fractions containing PheRS activity were collected, concentrated in an Amicon cell and charged on a Sephacryl S 200 column (length 1.5 m, diameter 2 cm) equilibrated in buffer A. The enzyme was further purified on two FPLC columns (Mono Q HR 5/5 and Phenylsuperose HR 5/5 from Pharmacia). The first column was developed with a linear concentration gradient of KCl from 0 to 500 mM, 20 mM Tris-HCl, pH 7.5, 1 mM  $MgCl_2$ , 0.1 mM EDTA, 1 mM mercaptoethanol, 10% glycerol. The second column was equilibrated in 50 mM potassium phosphate, 1.7 M ammonium sulfate and protein eluted with an ammonium sulfate gradient that decreased to 0 M. To purify PheRS from the overproducing *E. coli* cells, the two first DEAE-cellulose columns were used and were followed by a hydroxyapatite column (60 ml HA-Ultrogel, IBF) equilibrated in 25 mM potassium phosphate buffer containing 1 mM  $MgCl_2$ , 0.1 mM EDTA, 1 mM mercaptoethanol and 10% glycerol. The hydroxyapatite column was charged with the pooled active fractions of the second DEAE-cellulose column (dialysed against the starting buffer) and protein eluted with 400 ml of the equilibration buffer

containing increasing concentrations of potassium phosphate up to 300 mM.

### 2.4. Determination of the kinetic parameters of phenylalanyl-tRNA synthetase

The phenylalanine activation reaction was assayed by the [ $^{32}P$ ]pyrophosphate-ATP exchange as previously described [14]. Concentrations of phenylalanine for determination of the  $K_m$  were 0.02, 0.025, 0.05, 0.1, 0.2 and 0.5 mM. For the  $K_i$  determinations, the phenylalanine concentrations were 0.02, 0.05 and 0.3 mM with OTA concentrations of 1, 2 and 3 mM.

Aminoacylation activity was measured in the presence of pure *E. coli* tRNA<sup>Phe</sup> as described elsewhere [14]. The  $K_m$  value for phenylalanine was determined using 0.02, 0.025, 0.033, 0.05, 0.1 and 0.15 mM phenylalanine. The  $K_i$  was determined using phenylalanine concentrations of 0.02, 0.04 and 0.08 mM in the presence of 2, 3 and 5 mM OTA.

### 2.5. Determination of in vivo phenylalanine concentrations

Phenylalanine concentrations within the cells were determined by a previously described isotope dilution method [15]. Briefly, increasing volumes of non-dialysed crude extracts were added to the aminoacylation reaction mix (1.6 mM [ $^{14}C$ ]phenylalanine, 498 mCi/mM) and the different  $^{14}C$  activities of the tRNA<sup>Phe</sup> acylation plateaus were established. As controls, well-defined phenylalanine dilutions and crude extracts containing well-defined quantities of phenylalanine were used. The phenylalanine content was expressed in nmol per mg of crude extract protein or per liter of cell volume.

### 2.6. Determination of OTA concentrations in cells

After centrifugation, the cells were washed, sonicated in buffer A and the resulting crude extract diluted to a protein concentration of about 2 mg/ml. The OTA concentration in crude extracts of cells treated with this mycotoxin was determined spectrophotometrically at 380 nm ( $\epsilon_{380} = 14,700$ ). As control, defined quantities of OTA were added to crude extracts of non-treated cells and measured against a crude extract of non-treated cells. Alternatively, OTA was extracted

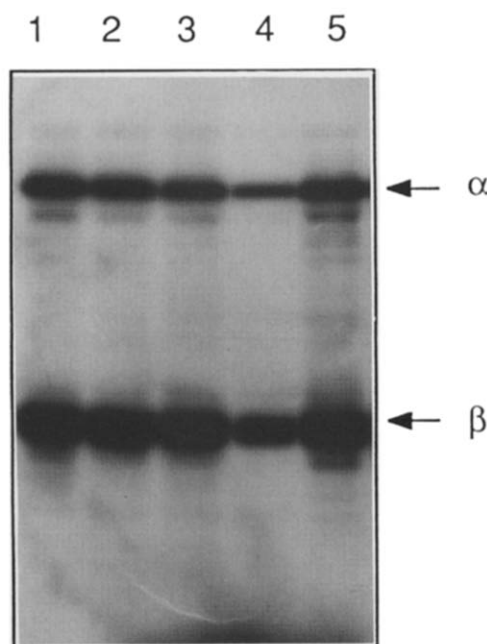


Fig. 2. Western blot analysis of an SDS-polyacrylamide gel of crude extracts from *B. subtilis* cells treated with 30  $\mu$ M OTA (1) 40  $\mu$ M OTA (2 and 3) without OTA (4) and of a culture of *B. subtilis* PSL 1 containing the shuttle vector pUC18-pBC 16-1-*pheS/T* leading to a 5-fold overproduction of PheRS [13].

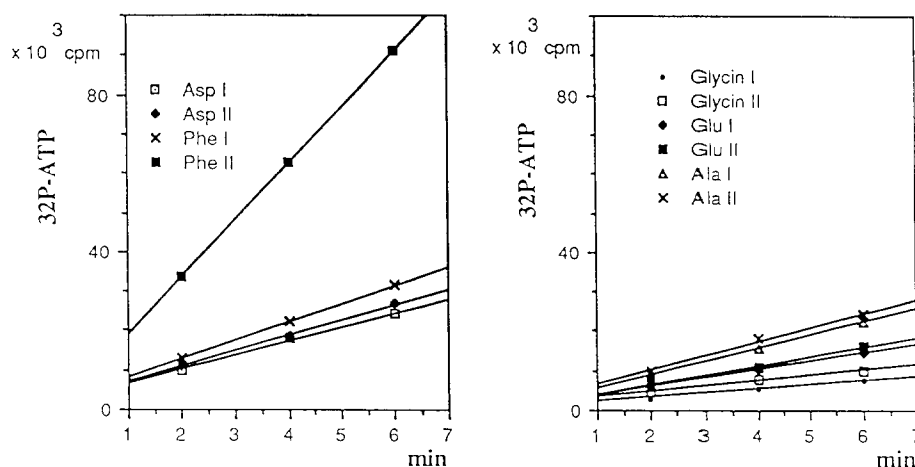


Fig. 3. The specific activities in the ATP-PP<sub>i</sub> exchange reaction catalysed by PheRS as well as other aminoacyl-tRNA synthetases (aspartyl-, glycyl-, glutamyl- and alanyl-tRNA synthetase) from *B. subtilis* cells treated with (II) and without (I) OTA.

out of these samples by chloroform and titrated spectrophotometrically.

#### 2.7. Anti-PheRS immunoglobulins

Anti-PheRS serum was obtained from rabbits which were immunized at 15-day intervals by three injections of 500 µg pure PheRS dissolved in 1 ml 10 mM phosphate buffer, pH 7, 14 mM NaCl and emulsified with 1 ml incomplete Freund's adjuvant. One week after the last injection, the rabbits were bled and serum was obtained by centrifugation of the blood.

#### 2.8. SDS-polyacrylamide gels and Western blotting

Proteins were separated on 10% polyacrylamide gels containing 0.1% SDS [16]. Western blot analysis was performed as described elsewhere [17].

#### 2.9. Isolation of ochratoxin A

OTA was isolated and purified from wheat cultures of *Aspergillus ochraceus* NRRL 3174 [18]. It was dissolved in 0.1 M NaHCO<sub>3</sub>. Its concentration was determined spectrophotometrically.

### 3. RESULTS

#### 3.1. Purification of PheRS from an overproducing strain

Phenylalanyl-tRNA synthetase from the wild type strain *B. subtilis* 168 was purified to homogeneity with a purification factor of about 600. It is an  $\alpha_2\beta_2$  tetramer of 270 kDa. PheRS is overproduced in *E. coli* JM109 transformed by bluescript-M13-*pheS/T*, as assayed by SDS-polyacrylamide gel electrophoresis (Fig. 1): there is clearly more protein migrating where the *B. subtilis* PheRS  $\alpha$  subunit (93,000 Da) and  $\beta$  subunit (41,000 Da) are found compared to the protein obtained from the wild type strain. Purification of the enzyme to homogeneity from the *E. coli* overproducing strain was achieved after two DEAE cellulose columns and a hydroxapatite column with a purification factor of about 15. This represents a 40-fold overproduction of PheRS in the *E. coli* transformant strain compared to the amount of enzyme obtained in *B. subtilis*.

#### 3.2. PheRS activity in OTA treated cells

The specific activity of PheRS is 3-fold higher in crude extracts from cells grown in the presence of OTA (30 and 40 µM) than in cells grown in its absence. Western blot analysis of crude extracts of *B. subtilis* cells treated with 30 and 40 µM OTA showed that OTA-grown cells have more of both subunits than does the crude extract of the control culture (Fig. 2). No significant activity increase was detected for other aminoacyl-tRNA synthetases we tested (Fig. 3). Thus, there is not a constitutive non-specific increase of all aminoacyl-tRNA synthetases due to modification of the growth conditions.

#### 3.3. Competition of OTA with phenylalanine for PheRS

OTA competitively inhibits the PheRS catalysed ATP-pyrophosphate exchange as well as the tRNA<sup>Phe</sup> charging reaction: the  $K_i$  values for OTA obtained from a Dixon plot representation were 0.8 mM and 4.33 mM, respectively, in the exchange (Fig. 4) and the aminoacylation (Fig. 5) reactions. The  $K_m$  for phenylalanine was 28 µM in both reactions. Thus, kinetic measurements on pure PheRS show that the inhibitory constant of this enzyme for OTA is more than 150 times higher than the  $K_m$  for its natural substrate.

Finally it has to be pointed out that OTA, in contrast to phenylalanine, is not activated by PheRS: no ATP-pyrophosphate exchange was observed with OTA in the absence of phenylalanine (results not shown).

### 4. DISCUSSION

Highly purified PheRS was obtained from genetically engineered *Bacillus subtilis* cells and its catalytic properties for its natural substrate and the phenylalanine analog, ochratoxin A were tested. The inhibitory constant of this enzyme for OTA (4.33 mM) is more than 150 times higher than its  $K_m$  for phenylalanine (28 µM) in

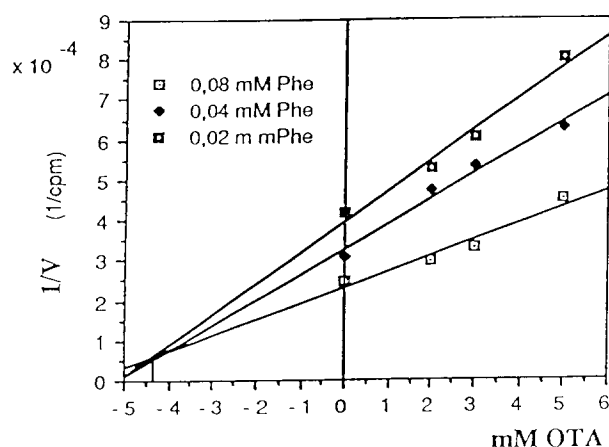


Fig. 4. Dixon plot representation of the initial rates of the [ $^{32}\text{P}$ ]pyrophosphate-ATP exchange reaction in the presence of different phenylalanine and OTA concentrations. It reveals a  $K_i$  value of 0.8 mM for OTA.

the aminoacylation reaction. These values are very close to those obtained for yeast PheRS ( $K_i$ : 1.3 mM,  $K_m$ : 3.3  $\mu\text{M}$ ) [19] but markedly different from those determined by Konrad and Rösenthaller for the *Bacillus subtilis* enzyme [11]. One reason for this difference may be that pure PheRS and *E. coli* tRNA<sup>Phe</sup> were used here. Another more likely explanation for such a discrepancy is the rather limiting aminoacylation conditions used by these authors: a weakly buffered reaction mixture (4 mM Tris-HCl, pH 7) and no free  $\text{Mg}^{2+}$  since equimolar concentrations of magnesium and ATP were used. Furthermore, initial reaction rates for  $K_m$  value determinations were estimated from kinetic curves systematically established from only one reaction point (7 min).

The growing of *B. subtilis* cells is inhibited by 20–40  $\mu\text{M}$  OTA concentrations [20] which are about 100–200 times lower than its  $K_i$  value in aminoacylation. Thus, the cell starvation effect cannot be explained by competitive inhibition of OTA unless this analog accumulates in the cells during growth. The OTA concentration in cells grown in the presence of 40  $\mu\text{M}$  OTA does reach 1 mM, as determined by absorption measurements of the corresponding crude extracts at 380 nm. This 25-fold enrichment reveals the existence of an active cell transport mechanism for OTA. The phenylalanine concentration is about 0.3 mM. In spite of the higher concentration of OTA in the cell compared to that of phenylalanine, it is difficult to explain the inhibition of protein synthesis by competition of this mycotoxin with phenylalanine for PheRS. The respective  $K_m$  and  $K_i$  values for these ligands do not favour PheRS as the direct target of OTA in the induction of cell starvation. However, only a demonstration that the charging level of tRNA<sup>Phe</sup> is unchanged *in vivo* by OTA treatment would definitively preclude this hypothesis.

Another mechanism must be responsible for the effect

of OTA on growing cells; if competition between OTA and phenylalanine is responsible, it must occur at a level different from PheRS. Several observations we made during these studies may contribute to understanding this starvation phenomenon. We observed that the toxic effect of OTA in *B. subtilis* cultures is less important when phenylalanine is present in the medium: cells cultured in minimal medium without phenylalanine stopped growing at concentrations of 10–20  $\mu\text{M}$  of OTA whereas cells cultured with 200  $\mu\text{M}$  phenylalanine stopped growing at concentrations of 20–40  $\mu\text{M}$  of OTA. This effect was not due to prevention of OTA uptake by phenylalanine entering since we found only a slightly lower concentration of OTA in cells grown in the presence of high concentrations of phenylalanine. This is in favour of a transport system for OTA which differs from that of phenylalanine. Furthermore, we observed that cells treated with OTA and cultured again after a certain period of starvation, contain 2–3 times higher PheRS activity compared to control cells. Western blot analysis demonstrated that this increase corresponds to accumulation of higher amounts of PheRS. It is well known that about half of the *E. coli* aminoacyl-tRNA synthetases (including PheRS) increase by a factor of 2–3 when cells are starved of amino acids [21]. The increase of PheRS occurs via an attenuation mechanism [22]. Thus, it was tempting to attribute the observed enzyme increase to OTA-induced phenylalanine starvation. However this hypothesis is not supported by the observation that no significant differences in phenylalanine concentrations could be measured between control and OTA-treated cells. Whatever the reasons for the increase in PheRS, they are secondary to the effect of OTA on cell growth.

**Acknowledgements:** We thank M.L. Gangloff for her skillful technical assistance. This work was supported by grants from the Ligue Nationale de la Recherche contre le Cancer, Comité Départemental du Haut-

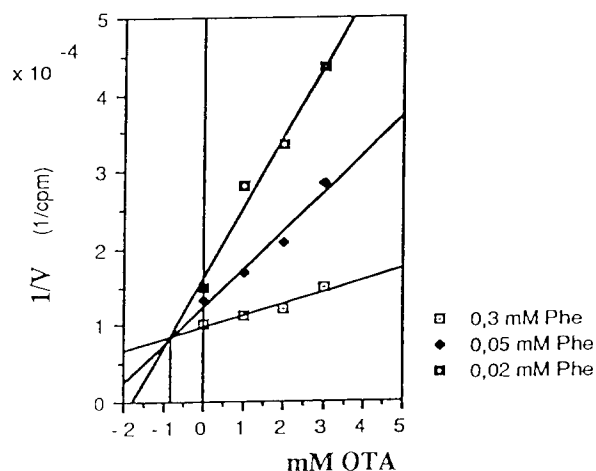


Fig. 5. Dixon plot representation of the initial rates of the tRNA<sup>Phe</sup> acylation reaction in the presence of different [ $^{14}\text{C}$ ]phenylalanine and OTA concentrations. It reveals a  $K_i$  value of 4.33 mM for OTA.

Rhin, from the Ministère de la Recherche et de la Technologie (action toxicologie) and from the Fondation pour la Recherche médicale.

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