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Biochemical basis for glyphosate-tolerance in a bacterium and a plant tissue culture

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The broad spectrum herbicide glyphosate (*N*-[phosphonomethyl]-glycine) is an inhibitor of the shikimate pathway enzyme 5-enolpyruvylshikimic acid-3-phosphate (EPSP)-synthase of both bacterial and higher plant origin. *Aerobacter aerogenes*, as well as cultured cells of the higher plant *Corydalis sempervirens*, adapted to growth in the presence of up to 10 mM glyphosate, exhibited a 10–30-fold increase in their EPSP-synthase activity, and excreted (*A. aerogenes*) or accumulated (*C. sempervirens*) massive amounts of shikimic acid-3-phosphate and/or shikimic acid.

Glyphosate *5-Enolpyruvylshikimic acid-3-phosphate synthase* *Enzyme inhibition* *Tolerance* *Adaptation*

1. INTRODUCTION

Single cells, as well as multicellular organisms, may potentially acquire resistance to metabolic inhibitors, such as those being used as antibiotics, drugs, or pesticides, by a number of different mechanisms. These include reduced uptake or increased detoxification of the inhibitor, a reduced affinity of the target site for binding the inhibitor, or an increase in the number of target sites as a consequence of their overproduction [1]. Overproduction of enzymes required for growth has been observed in mammalian cell lines which had been selected for their ability to grow in increasing concentrations of specific enzyme inhibitors [2–5]. In at least two cases, methotrexate and *N*-[phosphonoacetyl]aspartate resistance, enzyme overproduction has been found to be attributable to gene amplification [6]. Mechanisms underlying resistance to growth inhibitors in cultured plant cells are less understood [7]. In soybean and maize cell cultures, aminopterin resistance was associated with increased dihydrofolate reductase activity [8,9], and in cultured tobacco cells resistance to acetohydroxamate was correlated with high levels of urease [10].

We have recently shown that the broad spectrum, non-selective herbicide glyphosate (*N*-[phosphonomethyl]glycine) is an inhibitor of the shikimate pathway enzyme 5-enolpyruvylshikimic acid-3-phosphate (EPSP)-synthase (EC 2.5.1.19; the recently revised name of this enzyme is 3-phosphoshikimate-1-carboxyvinyltransferase) of the bacterium *Aerobacter aerogenes* [11]. The enzyme from this source has been purified to homogeneity and found to be competitively inhibited by glyphosate with respect to the substrate phosphoenolpyruvate [12,13]. EPSP-synthase activities in extracts from higher plants were, likewise, inhibited by glyphosate, and for the partially purified enzyme from cultured cells of *Corydalis sempervirens* competitive inhibition by glyphosate was confirmed ([13]; Amrhein, Leifeld and Steinrücken, in preparation). Inhibition in vivo of EPSP-synthase by glyphosate is evident from the fact that plants, and cultured plant cells,

Abbreviation: EPSP-synthase, 5-enolpyruvylshikimic acid-3-phosphate synthase (EC 2.5.1.19; 3-phosphoshikimate 1-carboxyvinyltransferase)

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accumulate massive amounts of shikimic acid and, to a lesser extent, shikimic acid-3-phosphate when exposed to glyphosate [13–16]. Here we report that adaptation to growth in the presence of glyphosate is associated with an increased EPSP-synthase activity in *A. aerogenes*, as well as in cultured cells of *C. sempervirens*.

2. MATERIALS AND METHODS

Aerobacter aerogenes (= *Klebsiella pneumoniae*), strain 1009/III, came originally from the laboratory of Professor Wallenfels (University of Freiburg) and was obtained from Professor Winkler (Ruhr University). The bacteria were grown at 37°C on a rotary shaker at 100 rev./min in the minimal medium used in [17] supplemented with 0.5% glucose, in 300-ml Ehrlenmeyer flasks containing 50 ml medium. Growth was followed by measuring the absorbance at 650 nm. For EPSP-synthase extraction, the cells from one flask were harvested by a 10 min centrifugation at 10000 × *g*, resuspended in ice-cold 1.5 ml extraction medium containing 0.2 M Tris–HCl buffer (pH 7.8), 5 mM mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (PMSF) and disrupted by sonication (Branson Sonifier B 12). After 15 min centrifugation at 10000 × *g* the supernatant was dialyzed overnight against the extraction medium (without PMSF). EPSP-synthase was determined as in [11]. Shikimic acid and shikimic acid-3-phosphate were determined in aliquots of the growth medium by the procedure previously described for shikimic acid [16]. Shikimic acid-3-phosphate eluted from the high pressure liquid chromatography (HPLC) column with a R_t of about 75 min [13].

The cell suspension culture of *Corydalis sempervirens* Pers., obtained from Professor Zenk (Munich University) was maintained in the medium used in [18] supplemented with 1 μM 2,4-dichlorophenoxyacetic acid and 1 μM 1-naphthylacetic acid. Cells (1.5 g fresh wt) were transferred to 100-ml Ehrlenmeyer flasks containing 25 ml medium, grown in continuous light (600 lux) at 100 rev./min and 23°C, harvested on a Buchner funnel and immediately frozen in liquid N_2. The frozen cells were allowed to thaw in an equal amount (v/w) of ice-cold 0.25 M Tris–maleate buffer (pH 7.7), containing 5 mM mercaptoethanol and 0.2 g Polyclar AT/g fresh wt, and ground in a mortar with some quartz sand. The homogenate was strained through cheesecloth and centrifuged at 12000 × *g* for 10 min. The supernatant was saturated to 80% with $(NH_4)_2SO_4$, the precipitate recovered by a 10 min centrifugation at 39000 × *g* and redissolved in a small volume of the extraction buffer. After overnight dialysis against 20 mM Tris–maleate buffer (pH 7.7) containing 10 mM mercaptoethanol, EPSP-synthase activity was determined as in [11], except that 50 mM Tris–maleate buffer (pH 7.7) and 5 mM substrate concentrations were used. The shikimic acid content of the cells was determined after methanolic extraction as in [16]. Protein concentrations in cell-free extracts were determined by the method in [19]. Analytical grade glyphosate (free acid) was provided by Monsanto Agricultural Products (St Louis MO).

3. RESULTS

Growth of *A. aerogenes* in the presence of glyphosate is affected in two ways: first, the lag-phase of growth is prolonged, and second, the growth rate is reduced (fig.1, table 1). In agreement with results previously obtained with *Escherichia coli* [20,21] and *Rhizobium japonicum* [22] a mixture of the 3 aromatic amino acids, plus *p*-hydroxybenzoic acid and *p*-aminobenzoic acid, which are all biosynthesized in bacteria from chorismic acid, reversed the growth inhibition (table 1). While neither shikimic acid nor shikimic acid-3-phosphate were found in the medium when bacteria were grown in the absence of glyphosate, both metabolites were excreted in the presence of glyphosate (table 1), which indicates in vivo inhibition of EPSP-synthase by glyphosate. Furthermore, the reduced formation of shikimic acid and its phosphorylated derivative in the presence of glyphosate plus the aromatic amino acids is in accord with the known regulation of the shikimate pathway in *A. aerogenes* [2,3]. Bacteria growing continuously in medium containing 5 mM glyphosate developed resistance to growth inhibition, and after 7 passages, the parameters of their growth in the presence of 5 mM glyphosate were almost indistinguishable from those of bacteria which were growing in the absence of glyphosate (fig.1, table 1). Glyphosate tolerance was parallel-

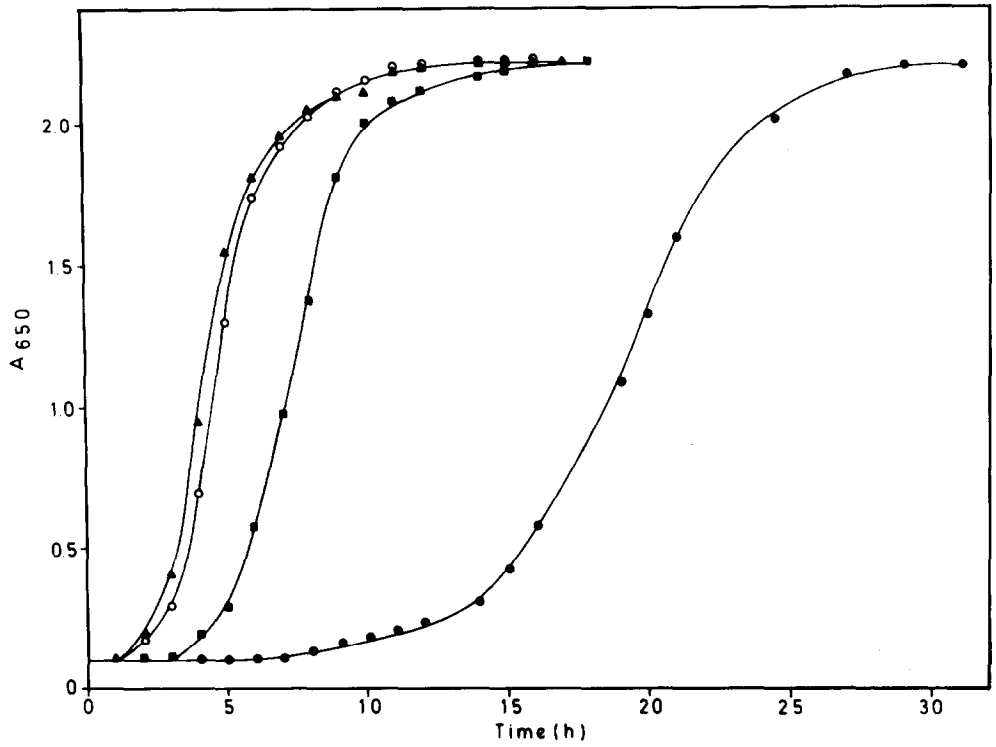

Fig.1. Effect of 1 mM (—■—) and 5 mM (—●—, —○—) glyphosate on the growth of *A. aerogenes*, which had been adapted (open symbols) or not adapted (closed symbols) to 5 mM glyphosate. Non-adapted cells in control medium: (—▲—).

Table 1

Growth parameters of *A. aerogenes*, either adapted or non-adapted to glyphosate, and excretion of shikimic acid (phosphate) under various conditions

Addition to minimal medium	Prolongation of lag-phase (h)	Generation time (min)	Relative growth[a] (% of control)	Concentration (μM) in medium[b]	
				Shikimic acid	Shikimic acid-3-phosphate
Non-adapted					
None (control)	0	54	100	0	0
1 mM Glyphosate	2	72	80	10	244
1 mM Glyphosate + supplement[c]	0	55	100	0	60
5 mM Glyphosate	12	138	0	190	655
5 mM Glyphosate + supplement[c]	0	60	98	105	495
Adapted[d]					
None	0	56	100	0	0
5 mM Glyphosate	0.2	59	98	215	696

[a] Calculated from period required to reach a cell density of $A_{650} = 0.5$

[b] Measured when bacteria were entering the stationary phase of growth

[c] Phe, Tyr, Trp each at 10 mg.l^{-1}, plus *p*-OH-benzoic acid and *p*-NH_2-benzoic acid, each at 10 μg.l^{-1}

[d] By 7 passages in the presence of 5 mM glyphosate

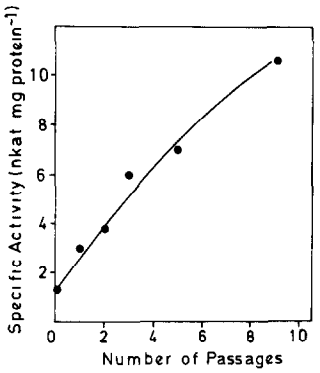

Fig.2. Effect of repeated transfer of *A. aerogenes* into growth medium containing 5 mM glyphosate on specific EPSP-synthase activity.

ed by an increased specific activity of EPSP-synthase in extracts prepared from the bacteria: after 9 passages the enzyme activity had increased nearly 10-fold (fig.2). The fact that bacteria which had adapted to glyphosate still excreted shikimic acid and its phosphate (table 1) shows that their EPSP-synthase is still sensitive to inhibition by glyphosate.

The cell culture of *C. sempervirens* was initially selected as a source of higher plant EPSP-synthase, because it produced the enzyme with higher specific activity than a number of other cultures which we tested. Also, it did not require exogenous amino acids (casein hydrolysate) for growth. The latter aspect was important for the selection of glyphosate tolerant cells, because the aromatic amino acids were known to reverse growth inhibition by glyphosate in plant cell cultures [20,23]. Under our culture conditions, cells growing in the absence of glyphosate required 8–9 days to enter the stationary phase of growth, and the total extractable EPSP-synthase activity reached a peak in the late logarithmic phase (fig.3). The specific activity of EPSP-synthase at this stage was 0.5 ± 0.1 nkat.mg^{-1} protein. When transferred to medium containing glyphosate at concentrations >0.5 mM, the cells did not proliferate at all. In 0.5 mM glyphosate, growth was severely inhibited for 10–14 days and was then resumed at a slow rate. After a total culture period of 4–5 weeks the cells were again transferred to medium containing 0.5 mM glyphosate, and, without following a strict temporal regimen, whenever a culture had

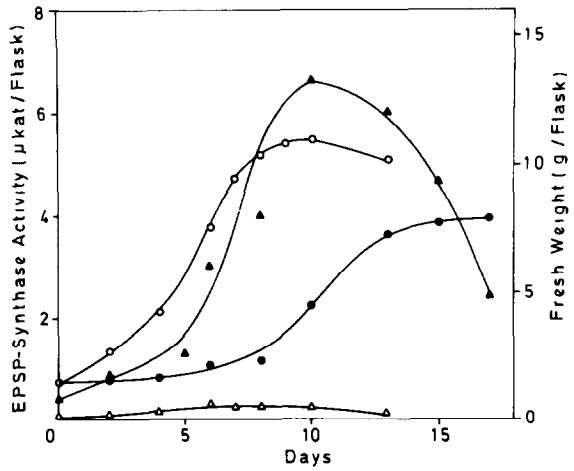

Fig.3. Fresh wt (—○—, —●—) and total EPSP-synthase activity (—△—, —▲—) during growth of *C. sempervirens* cells. Open symbols: non-adapted cells in control medium. Closed symbols: cells, adapted to 5 mM glyphosate, in medium containing 5 mM glyphosate.

grown up, cells were transferred to media containing increasing concentrations of glyphosate. Thus, within about 9 months, we had cells growing in glyphosate concentrations of up to 10 mM.

Table 2 lists the specific EPSP-synthase activities and shikimic acid levels of *C. sempervirens*

Table 2

EPSP-synthase activity and levels of shikimic acid in cultured cells of *C. sempervirens* continuously cultured in the presence of glyphosate[a]

Concentration of glyphosate in growth medium (mM)	EPSP-synthase activity (nkat·mg protein^{-1})	Shikimic acid (µmol·g fresh wt^{-1})
0	0.5 ± 1	0.18 ± 0.04
0.5	7.3 ± 2.5	7.88 ± 1.44
1	7.0 ± 3.7	8.84 ± 1.84
2	11.7 ± 4.0	10.03 ± 1.99
3	12.2 ± 3.8	10.06 ± 2.26
5	12.8 ± 2.7	15.74 ± 2.69
10	16.6 ± 3.0	18.00 ± 2.84

[a] Cultures were harvested when visual inspection showed that they had entered the stationary phase of growth (2–3 weeks after transfer, except for the controls). ± SD are given for 15–20 measurements during a 9–12 month period

cells which had been growing in the presence of the indicated glyphosate concentrations for at least a 9-month period. Measurements were made each time the cells were transferred to fresh medium; i.e., during the early stationary phase. Fig.3 compares the kinetics of growth and total EPSP-synthase activity of normal *C. sempervirens* cells in medium without glyphosate and of cells adapted to 5 mM glyphosate in medium with 5 mM glyphosate. It is evident that:

(i) the growth of the tolerant cells is considerably delayed;
(ii) the extractable activity of EPSP-synthase in these cells is greatly enhanced.

The approximate 30-fold increase in the specific EPSP-synthase activity in cells adapted to 10 mM glyphosate (table 2) is a conservative value, because the activities were determined in the stationary phase, when the specific activity had already begun to decline (not shown). This fact also explains the relatively large standard deviations, because, due to the cultures' slow entry into the stationary phase, it is likely that measurements were not always taken at the same physiological state of the cultures. Only shikimic acid, and not shikimic acid-3-phosphate, was found to accumulate in cells exposed to glyphosate (table 2). It is likely, from our previous experience with buckwheat cell cultures [16], that shikimate acid-3-phosphate, which cannot be further metabolized in the presence of glyphosate, is hydrolyzed to shikimic acid during uptake into, or in, the vacuole. Neither shikimic acid nor shikimic acid-3-phosphate were found in the media.

4. DISCUSSION

We have shown that a bacterium, *A. aerogenes*, as well as cultured cells of a higher plant, *C. sempervirens*, can adapt to increasing concentrations of the growth inhibitor glyphosate. We had previously demonstrated that glyphosate is a potent and specific inhibitor of *A. aerogenes* EPSP-synthase [11,12]. The combined facts that:

(i) *A. aerogenes* excretes shikimic acid-3-phosphate and, to a lesser extent, shikimic acid in the presence of glyphosate (table 1);
(ii) aromatic amino acids reverse the growth inhibition by glyphosate (table 1).
(iii) EPSP-synthase specific activity increases during continuous culture of the bacteria in the presence of glyphosate (fig.2);
(iv) bacteria possessing increased EPSP-synthase activity tolerate high concentrations of glyphosate (fig.1),

presents compelling evidence that growth inhibition of *A. aerogenes* by glyphosate is due to the inhibition of EPSP-synthase. In this context, the recent report of genetically engineered glyphosate tolerance in *E. coli* [25] is of great interest. It was shown here that glyphosate tolerance is related to increased levels of EPSP-synthase activity. In *C. sempervirens*, an adaptation to glyphosate, comparable to that occurring in *A. aerogenes*, is observed, but the time scale is greatly expanded. Nevertheless, we observed that during the initial exposure to 0.5 mM glyphosate, the specific EPSP-synthase activities of 10 different cloned cell lines of *C. sempervirens* increased by an average factor of 10 and thereafter increased more gradually during the following transfers (Amrhein and Schab, unpublished). This observation may explain the absence of a simple relationship between the concentration of glyphosate in which the cells were grown, and their specific EPSP-synthase activity (table 2). The comparably rapid initial increase in EPSP-synthase activity in response to glyphosate, as well as the uniform response of the cloned cell lines seems to exclude the possibility that we selected cells (either mutants or epigenetic variants) which possessed an initially elevated level of EPSP-synthase activity, and, likewise, gene amplification [6] is an unlikely mechanism to explain our observation. On the other hand, since we have as yet not found differences between the properties of EPSP-synthases isolated from control and glyphosate-tolerant cells (in particular with respect to inhibition by glyphosate) we assume, as a working hypothesis, that the increased EPSP-synthase activity is the result of enzyme overproduction.

As a welcome practical result of the present investigation we can now conveniently isolate and further purify EPSP-synthase to high specific activity from glyphosate-tolerant *C. sempervirens* cells (Amrhein and Johänning, unpublished), which will be of great value for the study of the mode of action of the herbicide.

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