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Regulatory Effects of Carbon Sources on Tabtoxin Production (A β-lactam Phytotoxin of *Pseudomonas syringae* pv. tabaci)

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***Abstract*—**The effects of divers carbon substrates were investigated for the tabtoxin production of an isolated pathogenic *Pseudomonas syringae* pv. tabaci, the causal agent of wildfire of tobacco and are discussed in relation to the bacterium growth. The isolated organism was grown in batch culture on Woolley's medium (28°C, 200 rpm, during 5 days). The growth has been measured by the optical density (OD) at 620 nm and the tabtoxin production quantified by *Escherichia coli* (K-12) bioassay technique. The growth and the tabtoxin production were both influenced by the substrates (sugars, amino acids, organic acids) used, each, as a sole carbon source and as a supplement for the same amino acids. The most significant quantities of tabtoxin were obtained in presence of some amino acids used as sole carbon source and/or as supplement.

***Keywords*—**Amino acid supplement, carbon substrates, batch culture, *Pseudomonas syringae* pv. tabaci.

I. Introduction

PSEUDOMONAS *syringae* is the largest taxonomic group within the genus *Pseudomonas*, where over 40 subspecies are divided into pathovars [1]. Nearly all phytopathogenic members of the *P. syringae* group produce a characteristic type of low molecular weight phytotoxins [2].

Plant diseases associated with tabtoxin-producing *P. syringae* are caused by several pathovars, including but not limited to the pathovars tabaci and coronafaciens [3], are usually characterized by chlorotic halos surrounding foliar lesions [4]. The production of an extracellular toxin by pathovar tabaci, causal agent of wildfire disease of tobacco (*Nicotiana tabacum*), was first reported in 1925 [5]. Clayton [3] later tested its host range and showed that tabtoxin was not host specific. Produced initially as an inactive dipeptide precursor, tabtoxin, a monocyclic β-lactam antibiotic [6], undergoes hydrolysis by aminopeptidases to yield the active form called tabtoxinine-β-lactam (TβL) [7].

TβL irreversibly inhibits glutamine synthetase and leads to toxic accumulation of ammonia to cause chlorosis [8]. The activity of TβL is lost upon the opening of the β-lactam ring following exposure to β-lactamase to yield tabtoxinine [9].

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The biosynthetic precursors of tabtoxin consist of L-threonine and L-aspartate for the side chain and pyruvic acid and the methyl group of L-methionine for the β-lactam moiety. Tabtoxinine-β-lactam biosynthesis proceeds along lysine pathway until an unknown intermediate is reached. Previous studies have suggested that tabtoxin biosynthesis branched off from the lysine biosynthetic pathway before the formation of 2,6-diaminopimelic acid [10].

Through Tn5 mutagenesis and clonage procedures, involving *P. syringae* BR2B, the causal agent of bean wildfire, the chromosomal region encodes tabtoxin biosynthesis was identified to de approximately of 25 to 30 kb in length and contains all genetic informations necessary for the production of and resistance to tabtoxin [11]. No mutations affecting TβL were found outside this region [12]. Tree genes, *tabA, tabB* and *tblA*, essential for tabtoxin production have been characterized within tabtoxin biosynthesis region [13].

Carbon catabolite regulation is exerted at gene transcription level for several antibiotics [14].

The regulatory effects of carbon sources on toxin biosynthesis in plant pathogenic pseudomonads are poorly defined. The understanding of these restrictions may enable further clarification of the role of tabtoxin in disease development and maybe the possibility of the isolation of metabolic intermediate of medical interest.

II. Materials And Methods

A. Bacterial Strains and Media

The phytopathogenic *Pseudomonas syringae* pv. tabaci used is a wild strain isolated from an east region of Algeria. The *Escherichia coli* K-12 strain was used for the bioassay technique.

Strains were routinely cultured on nutrient broth-yeast extract (NBY) agar and *Pseudomonas* minimal medium (PMS) [15].

The basal medium used for tabtoxin production in batch cultures is a minimal medium with 1% sucrose as sole carbon source [16]. The ingredients were added from the following sterilized stock solutions (w/v): sucrose, 10%; $MgSO_4 .7H_2O$, 0.2 %; $FeSO_4 .7H_2O$, 0.02%; $CaCl_2 . 2H_2O$, 0.1%; KNO_3, 5%; K_2HPO_4, 0.8%; $NaH_2PO_4 . H_2O$, 0.8%.

The effects of various carbon sources were tested by replacing the sucrose in the basal medium with:

(Carbohydrates: fructose, galactose, glucose, glycerol, lactose, maltose, mannose, raffinose, ribose, sucrose; Organic Acids: citric acid, fumaric acid, acide lactique, propionic acid, salicylic acid, tartric acid; Amino acids: alanine, aspartic acid, glycine, leucine, methionine, proline, serine, valine).

Molarities were adjusted to 29.214 mM to keep the amount of carbon per liter constant between treatments.

The same amino acids used as sole carbon source were also added to the basal medium as a supplement at 3 mM [17]. The amino acids were dissolved in sterile distilled water and added by filtration.

The effects of carbon concentration were tested by adjusting the sucrose concentrations at 1/5, 1/2, 1, 2 and 5 times the basal medium amounts [17].

B. Growth conditions and measurement

Cells of isolated *P. syringae* pv. tabaci from NBY broth of 24 hours were grown in dark to exponential growth phase (1 to 5 × 10^8 UFC/ml) [18] in 100 flask containing 50 ml of Woolley's medium at 28°C and 200 rpm [19], and collected by centrifugation for 15 min at 11700 g and suspended in sterile distilled water. The bacterial suspension was used to inoculate the media to have a final concentration of between 6 to 9 × 10^7 UFC/ml. Cultures volumes of 100 ml contained in 250 ml flasks were incubating at 28°C for 5 to 6 days in dark with agitation of 200 rpm.

Bacterial growth was determined by cell density at 620 nm.

C. Bioassay for tabtoxin

This is based on the overlay technique which used *E. coli* K 12 as a sensitive organism. This organism was grown to log phase in shaken nutrient broth at 30°C and harvested by centrifugation (10000 g, 10 min, 4°C). The pellet was washed and a suspension (A500 nm = 0.2) was made in sterile saline. An equal volume of cooled (45°C) molten agar (0.7 %) was mixed with the *E. coli* suspension and 5 ml of the mixture was poured into a Petri dish containing 20 ml solid PMS medium [20]. The quantitative bioassay for tabtoxin was made by dispensing 0.1 ml of supernatant in wells cut through overlaid solid medium with a sterile corkborer (6 mm diameter). The cultures were incubated at 37°C for 18 hours. The zone of inhibited growth, measured around each well, represents tabtoxin quantity in millimeters.

III. Results And Discussions

Tabtoxin production on Woolley's medium at 28°C is partially associated with the growth of the isolated *P. syringae* pv. tabaci (Fig. 1). Tabtoxin production was absent during the first day of incubation, reached its maximum with an inhibition diameter of 9 mm at the beginning of the deceleration phase with a growth of 1.26 × 10^8 UFC/ml, remained constant till the end of the deceleration phase with a growth that remains < 2 × 10^8 UFC/ml, maybe due to an equilibrium between its production and its isomerization. Tabtoxin production decreased during the third day of incubation, then was absent in the fourth and fifth day of incubation.

These results are comparable to those found by Taylor and Durbin [21] in 1973 on tabtoxin production on Woolley's medium by a timothy pathogenic *Pseudomonas* sp. incubated at 24°C, where a small amount of tabtoxin is produced during log phase; however most is formed during the deceleration phase. This comparison reveals that temperature may be a secondary factor in tabtoxin production. Tabtoxin was also absent in the fourth and fifth day of incubation. Taylor and Durbin concluded that this is due to tabtoxin isomerization then to direct isotabtoxins production.

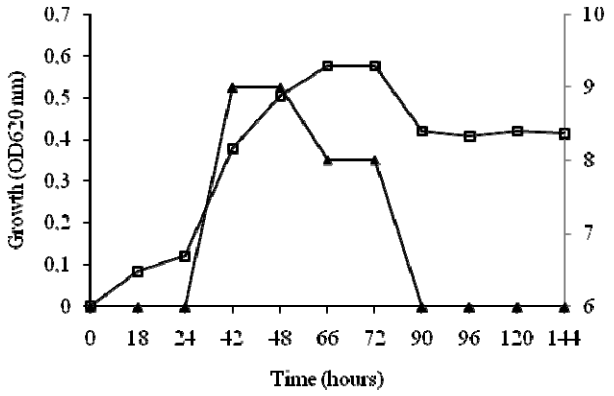

Fig. 1 Growth of *P. syringae* pv. tabaci □ and tabtoxin production ▲ in Woolley's medium.

A. Effects of carbon substrates as sole carbon source

1. Sugars

All media containing different carbohydrates as sole carbon source supported growth of the isolated *P. syringae* pv. tabaci with a less important growth rate in presence of ribose and fructose without exceeding 6 × 10^7 UFC/ml at the end of the log phase (Fig. 2 a). However, tabtoxin is absent in presence of fructose, mannose, lactose and ribose (Fig. 2 b). They may lessen or inhibit the synthesis of tabtoxin, or stimulate the production of inactive isotabtoxins.

The production of tabtoxin in the first day of incubation in presence of glucose and raffinose, in the second day in presence of galactose, maltose and saccharose, and till the third day in presence of glycerol shows that tabtoxin production is associated to different growth phases according to the carbohydrate used as sole carbon source.

With an inhibiting diameter of between 9-11 mm, the amount of tabtoxin produced is not significantly different between the media and thus the type of carbohydrate (mono, di-, tri disaccharide or alcohol) used didn't affect significantly the amount of tabtoxin. The sugars then favorites cell growth on tabtoxin production. May be these weak amounts of tabtoxin are due to metabolic intermediates generated by the sugars catabolic pathways and that interfere with tabtoxin synthesis pathway.

The tabtoxin production is absent at the fourth and fifth day of incubation.

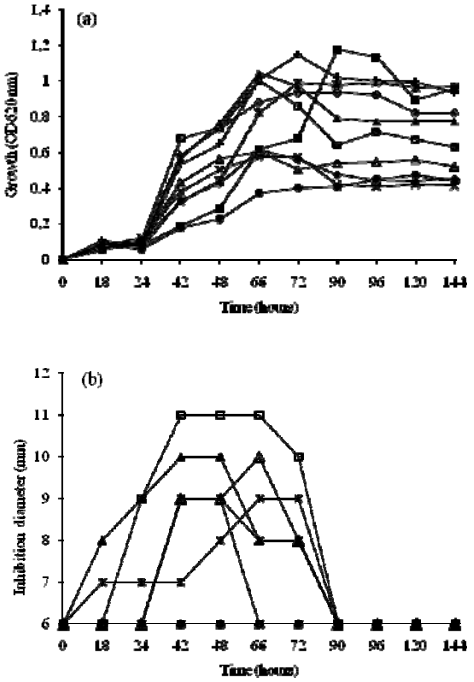

Fig. 2 Growth of *P. syringae* pv. tabaci (a) and tabtoxin production (b) in presence of different carbohydrates. Fructose ■, galactose □, glucose ▲, glycerol △, lactose ◆, maltose ◇, mannose +, raffinose ∗, ribose ●, sucrose ×.

2. Organic acids

All media containing each organic acid as sole carbon source supported growth of the isolated *P. syringae* pv. tabaci and all, allowed tabtoxin production (Fig. 2). With cell a number inferior to 10^8 UFC/ml during all growth phases, the growth is less important than obtained in presence of sugars. However, the amounts of tabtoxin produced in presence of tested organic acids are more important with inhibition diameters of between 9 and 18 mm and a longer idiophase going from the first to the fourth day of incubation (Fig. 2 b).

With inhibition diameter of 12 and 13 mm in presence of the two intermediates of Kreps cycle (KC), citrate and fumarate respectively, tabtoxin produced amounts are less than in presence of salicylate and tartrate with inhibition diameters of 17 and 18 mm respectively and more important than in presence of lactate and propionate that gave, as in presence of carbohydrates, the smallest inhibition diameter of 9 mm.

The catabolism of propionate and lactate returns to KC and glycolysis *via* succinyl CoA and pyruvate respectively, and the one of salicylate and tartrate returns to KC or glycolysis *via* these two points. This suggests that each metabolic pathway regulates tabtoxin production at definite amounts and that the carbon substrates having a common metabolic pathway act as effectors that reduce or increase these amounts.

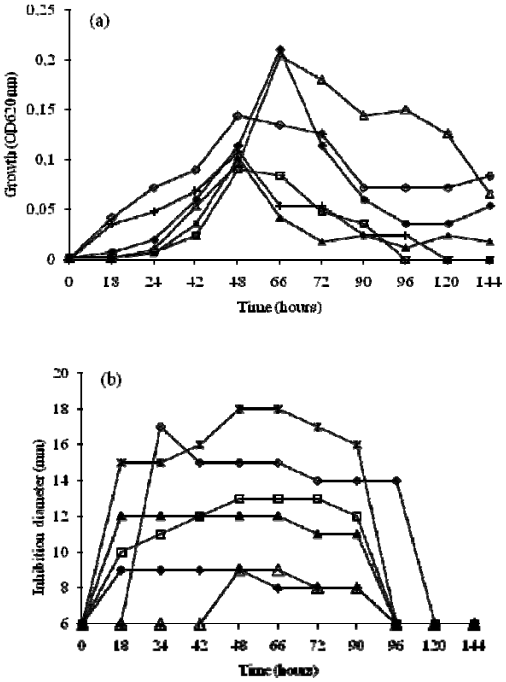

Fig. 3 Growth of *P. syringae* pv. tabaci (a) and tabtoxin production (b) in presence of different organic acids. Citrate □, fumarate ▲, lactate △, propionate ◆, salycilate ◇, tartrate ∗.

3. Amino acids

All the amino acids used as sole carbon source supported the growth of the isolated *P. syringae* pv. tabaci. Production of tabtoxin is only measured in presence of aspartate, leucine and serine with tabtoxin amounts three times more important than in presence of carbohydrates which remained constant during the five days of incubation (Fig. 4).

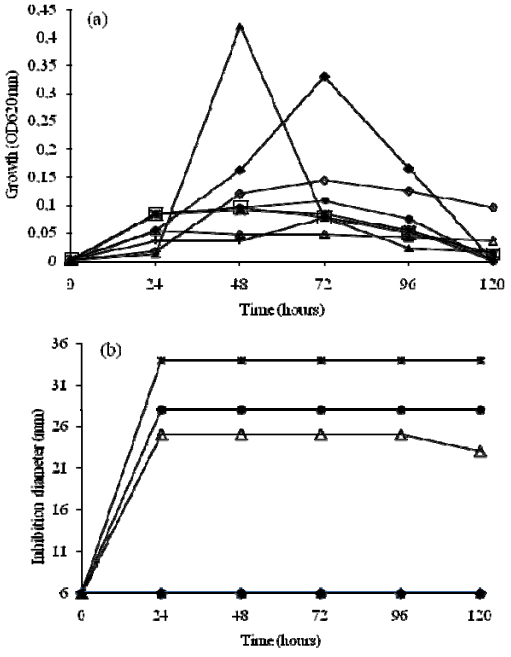

Fig. 4 Growth of *P. syringae* pv. tabaci (a) and tabtoxin production (b) in presence of different amino acids. Alanine ▲, aspartate △, glycine □, L-leucine∗, L-proline ◇, L-methionine +, serine ●, valine ◆.

The catabolic pathways of serine, leucine and aspartate return to carbon metabolism *via* pyruvate, acetyl CoA, and KC respectively. This suggests that they don't act only as

substrates that increase tabtoxin production but as positive signal molecules that stimulate tabtoxin production at very high amounts. In the opposite, the remaining amino acids may have a negative regulation on tabtoxin production. This

B. Effects of amino acids supplement

If the growth of the isolated *P. syringae* pv. tabaci is more important in the basal medium containing no amino acid supplement (Fig. 5a), tabtoxin produced amounts are two and three times more important with a supplement of 3 mM of aspartate, methionine and serine, then for the basal medium (Fig. 5b). This confirms that amino acids increase tabtoxin production not only as substrates but as signal molecules that stimulate the production of higher amounts of tabtoxin.

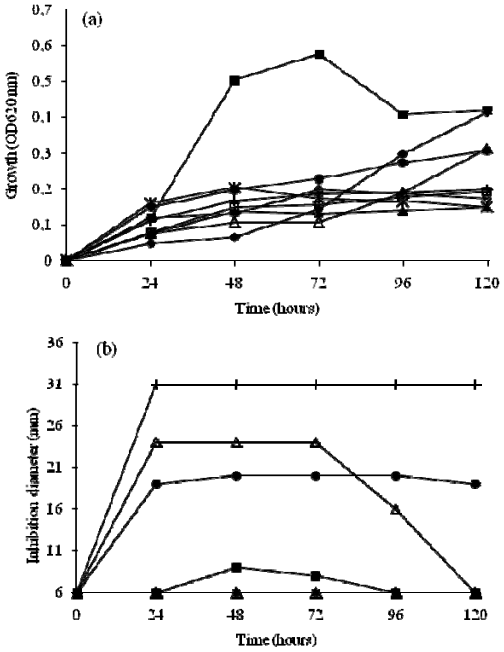

Fig. 5 Growth of *P. syringae* pv. tabaci (a) and tabtoxin production (b) on Woolly's medium supplemented by various amino acids. Alanine ▲, aspartate △, glycine □, L-leucine∗, L-proline ◇, L-methionine +, serine ●, valine ◆, control ■.

The decrease of tabtoxin production in presence of aspartate and leucine when used as a supplement comparing to their use as a sole carbon sources is probably due to their concentrations in the culture medium. However the decrease is not the same for the two amino acids suggesting that each one work on different regulatory mechanism. The absence of tabtoxin production when serine is used as a supplement may also be because of its concentration which is for this amino acid insufficient to stimulate tabtoxin production. This reinforce that the each amino acid has different regulatory effect. However the opposite is observed for methionine. This signify that methionine stimulate tabtoxin production at low concentration and that used with higher concentration (as substrate) it blocs tabtoxin production.

C. Effects of carbon concentrations

With carbon concentrations of between 1/2 and 5 times the initial concentration in the basal medium, the production of tabtoxin was not significantly different with inhibition diameters of 11 and 12 mm. The most important tabtoxin amount was measured in presence of 1/5 the initial carbon concentration with inhibition diameter of 15 mm (Fig. 6 b), suggesting that the absence of tabtoxin in presence of carbon substrate may be due to the inhibitory concentration for some or all of them.

The weakest growth in presence of 1/5 the initial carbon concentration corresponded to a highest amount of tabtoxin (15 mm), suggests that the feeble tabtoxine concentrations in presence of sugars may be ameliorated with a very low carbon concentration.

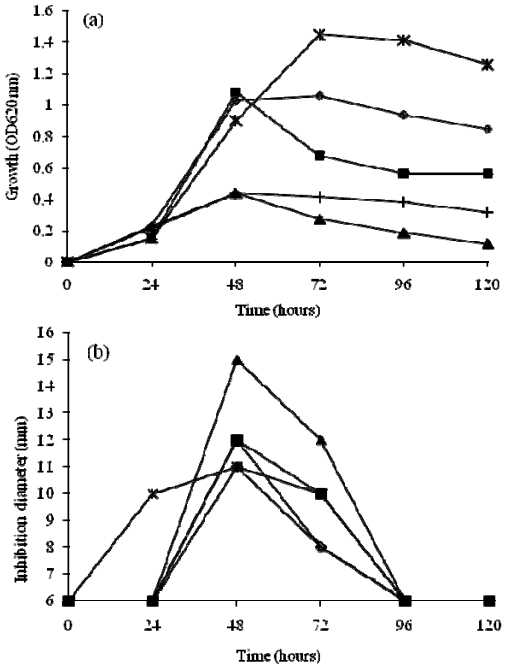

Fig. 6 Growth of *P. syringae* pv. tabaci (a) and tabtoxin production (b) in presence of different carbon (sucrose) concentration. C/5 ▲, C/2 ■, C ∗, 2C ○, 5C ◇.

IV. Conclusions

The growth and tabtoxin production were both regulated by carbon sources (sugars, amino acids, organic acids), by the amino acid supplement and carbon concentrations. Sugars were better substrates for growth then organic acids, then amino acids, whereas this order is inversed for tabtoxin production showing that primary catabolic metabolism regulates tabtoxin production.

The most significant quantities of tabtoxin were obtained in presence of some amino acids used as sole carbon sources and/or as supplements suggesting that amino acids may also work as positive or negative signal molecules in tabtoxin production.

Carbon sources regulated also the period and the idiophase duration probably through tabtoxin isomerization.

It will be interesting to investigate other carbon sources and lowest concentrations for tabtoxine production, but also the effect of the present sources on tabtoxin biosynthesis genes production and expression.

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