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Indole-3-acetic acid (IAA) synthesis in the biocontrol strain CHA0 of *Pseudomonas fluorescens*: role of tryptophan side chain oxidase

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Pseudomonas fluorescens **strain CHA0 is an effective biocontrol agent against soil-borne fungal plant pathogens. In this study, indole-3-acetic acid (IAA) biosynthesis in strain CHA0 was investigated. Two key enzyme activities were found to be involved: tryptophan side chain oxidase (TSO) and tryptophan transaminase. TSO was induced in the stationary growth phase. By fractionation of a cell extract of strain CHA0 on DEAE-Sepharose, two distinct peaks of constitutive tryptophan transaminase activity were detected. A pathway leading from tryptophan to IAA via indole-3-acetamide, which occurs in *Pseudomonas syringae* subsp. *savastanoi*, was not present in strain CHA0. IAA synthesis accounted for $\leqslant 1{\cdot}5\%$ of exogenous tryptophan consumed by resting cells of strain CHA0, indicating that the bulk of tryptophan was catabolized via yet another pathway involving anthranilic acid as an intermediate. Strain CHA750, a mutant lacking TSO activity, was obtained after Tn*5* mutagenesis of strain CHA0. In liquid cultures (pH 6·8) supplemented with 10 mM-L-tryptophan, growing cells of strains CHA0 and CHA750 synthesized the same amount of IAA, presumably using the tryptophan transaminase pathway. In contrast, resting cells of strain CHA750 produced five times less IAA in a buffer (pH 6·0) containing 1 mM-L-tryptophan than did resting cells of the wild-type, illustrating the major contribution of TSO to IAA synthesis under these conditions. In artificial soils at pH ~7 or pH ~6, both strains had similar abilities to suppress take-all disease of wheat or black root rot of tobacco. This suggests that TSO-dependent IAA synthesis is not essential for disease suppression.**

Introduction

Pseudomonas fluorescens strain CHA0 suppresses a variety of root diseases caused by soil-borne fungal pathogens (Stutz *et al.*, 1986; Défago *et al.*, 1990) and produces several secondary metabolites, notably HCN, 2,4-diacetylphloroglucinol, pyoluteorin and indole-3-acetic acid (IAA) (Défago *et al.*, 1990; Défago & Haas, 1990; Haas *et al.*, 1991). HCN has been shown to be an important factor in the suppression of black root rot of tobacco, a disease caused by *Thielaviopsis basicola* (Voisard *et al.*, 1989), and 2,4-diacetylphloroglucinol contributes significantly to the suppression of both black root rot of tobacco and take-all of wheat, a disease caused by *Gaeumannomyces graminis* var. *tritici* (Keel *et al.*, 1990, 1991). Many plant-beneficial *Pseudomonas* strains, isolated from the rhizosphere, produce IAA (Loper & Schroth, 1986; Müller *et al.* 1989). In bacteria, several pathways have been described leading from L-tryptophan (Trp) to IAA (Fig. 1). In a strain of *P. fluorescens* (ATCC 29574), one of these pathways is initiated by tryptophan side chain oxidase (TSO) (Narumiya *et al.*, 1979). An IAA biosynthetic pathway involving indolepyruvic acid as an intermediate occurs in *Agrobacterium tumefaciens* (Kaper & Veldstra, 1958) and in a bacterium isolated from the rhizosphere of *Festuca octoflora* (Frankenberger & Poth, 1988). In *Pseudomonas syringae* subsp. *savastanoi*, the olive and oleander knot pathogen, the biosynthesis of IAA via indolepyruvic acid is of minor significance compared to the formation of IAA via indoleacetamide (Kuo & Kosuge, 1970).

Here we report two pathways of *Pseudomonas fluorescens* strain CHA0 contributing to IAA production and we tested the influence of different incubation conditions on the amount of IAA produced. By isolating a Tn*5* insertion mutant lacking TSO, we could assess the contribution of this enzyme to IAA formation and to disease suppression.

Abbreviations: IAA, indole-3-acetic acid; KB, King's medium B; MES, 2-morpholinoethanesulphonic acid; NA, nutrient agar; NYB, nutrient yeast broth; OS medium, Ornston & Stanier medium; TSO, tryptophan side chain oxidase.

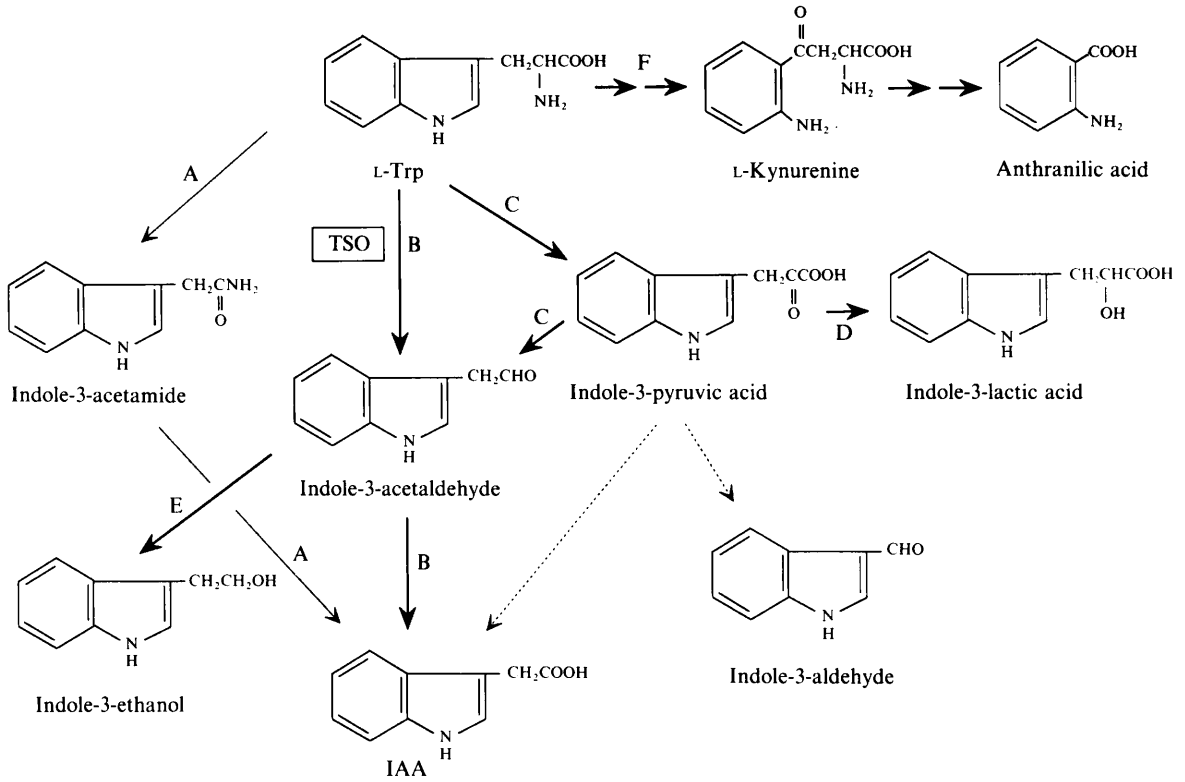

Fig. 1. Bacterial pathways leading from Trp to IAA: A, indoleacetamide pathway of *P. syringae* subsp. *savastanoi*, catalysed by Trp-2-mono-oxygenase and indoleacetamide hydrolase (Comai & Kosuge, 1980); B, TSO and indoleacetaldehyde dehydrogenase-catalysed formation of IAA (Narumiya *et al.*, 1979) [in addition to indoleacetaldehyde, TSO also produces indoleglycolaldehyde and indoleglyoxal (not shown; Narumiya *et al.*, 1979)]; C, indolepyruvic acid pathway initiated by L-Trp transaminase (Frankenberger & Poth, 1988); D, enzymic formation of indolelactate (Kaper & Veldstra, 1958); E, indole-ethanol dehydrogenase (Narumiya *et al.*, 1979); F, ring cleavage pathway initiated by Trp-2,3-dioxygenase leading to kynurenine and anthranilic acid (Narumiya *et al.*, 1979). Pathways of strain CHA0 are indicated with bold arrows (reactions B, C, D, E, F). Indolepyruvic acid is unstable and gives rise to a non-enzymic formation of IAA and indolealdehyde (Kaper & Veldstra, 1958).

Methods

Micro-organisms and growth conditions. The strains used were *Pseudomonas fluorescens* strain CHA0 (Stutz *et al.*, 1986) and its Trp auxotrophic derivative CHA104 (*trp-104*::Tn*5*; obtained from C. Voisard, Department of Microbiology, Swiss Federal Institute of Technology, Zürich, Switzerland), *Pseudomonas savastanoi* strain 229 isolated from *Nerium oleander* (Wädenswil, Switzerland), *Thielaviopsis basicola* (Berk. and Br.) Ferraris ETH strain D127, and *Gaeumannomyces graminis* (Sacc.) Olivier and Von Arx var. *tritici* Walker ETH strain 1000. The *Pseudomonas* strains were grown on King's medium B (KB) agar (King *et al.*, 1954), nutrient agar (NA) (Stanisich & Holloway, 1972), and in nutrient yeast broth (NYB); NYB contained (l^{-1}) 25 g nutrient broth and 5 g yeast extract (both Difco). NA and NYB were supplemented with kanamycin sulphate (25 μg ml^{-1}) for the selection of Tn*5* derivatives of strain CHA0. For enzyme determinations and IAA measurements the bacteria were grown on a rotary shaker (120 r.p.m.) at 27 °C in 500 ml Erlenmeyer flasks containing 200 ml minimal medium OS with 5 g glucose l^{-1} as the carbon source (Ornston & Stanier, 1966). In some cases L-Trp (sterilized by filtration) was added to a final concentration of 10 mM. *T. basicola* and *G. graminis* var. *tritici* were grown on malt agar and millet, respectively, at 24 °C (Keel *et al.*, 1989, 1991).

Preparation of cell extracts. Cells grown in minimal medium OS with or without 10 mM-L-Trp were harvested by centrifugation at 4 °C after 16 h (exponential phase) or 36 h (stationary phase), washed twice with ice-cold potassium phosphate buffer (100 mM, pH 7·5) and resuspended in the same buffer to obtain a 15% (w/v) suspension. Cells were disrupted with an ultrasonic homogenizer (Braun Labsonic 2000) by 5 pulses of 10 s, and cell debris was sedimented at 50000 *g* at 4 °C for 20 min. The crude cell extract was used directly for enzyme assays. For chromatography on DEAE-Sepharose and for transaminase assays, the cell extract was desalted on a Sephadex G-25 column (PD-10, Pharmacia) which was equilibrated with a 25 mM-potassium phosphate buffer (pH 7·5).

Enzyme assays. (*a*) *Trp transaminase.* The reaction mixture consisted of L-Trp and 2-oxoglutarate (5 mM each), 50 μM-pyridoxal phosphate, 0·5 mM-sodium arsenate and 0·5 mM-EDTA in 50 mM-borate/HCl buffer (pH 8·5). The formation of indolepyruvate as a stable enol tautomer–borate complex was measured as the increase in A_{327} at 25 °C (Frankenberger & Poth, 1988). The concentration of indolepyruvate was calculated from a standard curve of indolepyruvic acid (Fluka) in borate buffer containing EDTA and sodium arsenate as above, and an ε_{mM} of 17·85 cm^{-1} was obtained.

(*b*) *Phenylalanine transaminase* was measured in the same reaction mixture as above but with 5 mM-L-phenylalanine (L-Phe) instead of L-Trp. The formation of phenylpyruvate was determined by measuring A_{300}, using an ε_{mM} of 9·15 cm^{-1} (Fujioka *et al.*, 1970).

(*c*) *Trp-2-mono-oxygenase* and *indoleacetamide hydrolase* were measured according to Comai & Kosuge (1980). The reaction products indoleacetamide and IAA were quantified by HPLC (see below). *Pseudomonas savastanoi* strain 227 served as a reference.

(*d*) *TSO*. The formation of *N*-acetyl-α,β-didehydrotryptophanamide was measured as the increase in A_{333} at 25 °C with 1 mM-*N*-acetyl-L-tryptophanamide (Sigma) as substrate in 50 mM-potassium phosphate buffer (pH 6·0) (Narumiya *et al.* 1979).

(*e*) *Indoleacetaldehyde dehydrogenase* was measured according to Fujioka *et al.* (1970), as the A_{340} increase due to NADH formation.

One unit of the enzyme activities described above is defined as the formation of 1 μmol product min^{-1}. The enzyme activities determined are average values of at least two independent experiments.

Fractionation on DEAE-Sepharose. Desalted cell extract (5 ml) was applied to a DEAE-Sepharose column (CL-6B, Pharmacia; 1·6 × 11 cm) equilibrated with 25 mM-potassium phosphate buffer (pH 7·5). Elution was performed over a period of 11 h with a linear gradient formed by mixing 25 mM-potassium phosphate (pH 7·5) with 100 mM-potassium phosphate (pH 7·5) containing 0·5 M-NaCl. The flow rate was 13·5 ml h^{-1} and fractions of 2·7 ml were collected. Trp transaminase was eluted in fractions 27–32 and 49–53.

Protein determination. Protein concentration was determined by the Bio-Rad Protein Microassay according to Bradford (1976) with bovine serum albumin as standard.

IAA determination. Bacterial IAA production was measured either in liquid OS cultures with or without 10 mM-L-Trp at 24, 48 and 72 h, or in suspensions of resting cells. Resting cells were harvested from NYB cultures at 36 h, in the stationary phase of growth, by centrifugation at 4000 *g* for 20 min. The cells were washed twice with 0·01 M-potassium phosphate buffer (pH 6·5) and resuspended in 0·1 M-potassium phosphate buffer (pH 6 or 7) or in 0·1 M-2-morpholinoethanesulphonic acid (MES)/potassium phosphate buffer (pH 5·5), respectively, to a concentration of approximately 1·5 × 10^9 c.f.u. ml^{-1}. L-Trp was added to a final concentration of 1 mM. IAA production was measured in the supernatant during incubation on a gyratory shaker (120 r.p.m.) at room temperature over a period of 6 h.

For IAA extraction, 10 ml bacterial suspension was centrifuged at 4000 *g* for 15 min and the supernatant was acidified to pH 2·5–3·0 with HCl. Apolar compounds were extracted wtih a Waters Sep-Pack C-18 cartridge (Sandberg *et al.*, 1987), eluted with methanol, reduced to dryness and redissolved in 0·5 ml 65% (v/v) methanol for HPLC, or in 0·2 ml ethyl acetate for TLC. The HPLC system (Hewlett Packard 1090L) used a reversed-phase Nucleosil 120-5C_8 column (250 × 4 mm; Macherey and Nagel, Oensingen, Switzerland), which was thermostatically controlled at 45 °C. Injection volume was 1–10 μl and elution was performed with increasing concentrations of methanol in 0·025% ethanolamine/water acidified with phosphoric acid to pH 2·7. A two-step linear gradient of 20–42% methanol (0–8 min) and 42–46% methanol (8–15 min) was used. The flow rate was 1 ml min^{-1}. Compounds were detected in series by a UV diode array detector at 280, 300 and 327 nm, and a fluorescence detector (Shimadzu RF-510LC; excitation: 280 nm, emission: 360 nm). Integration of peak areas was performed with the 'ChemStation'-software (Hewlett Packard).

For TLC, 20–100 μl of the extracts was applied on silica plates and chromatographed with chloroform/ethyl acetate/formic acid (5:4:1, by vol.). The separated compounds were visualized by colour development with Ehrlich reagent (Hartmann *et al.*, 1983).

Transposon insertion mutagenesis. This was performed as described by Voisard *et al.* (1989) with strain CHA0 as the recipient and *Escherichia coli* strain W3110(pLG221) as the donor. TSO activity of the mutants obtained was detected by overlaying 4-d-old colonies on KB plates with agar (1·2%, w/v) containing 10 mM-L-Trp and 0·5% SDS in 0·5 M-glycine/HCl buffer at pH 3 (Takai & Hayaishi, 1987). Colonies of TSO-positive strains turned dark upon incubation at room temperature overnight, whereas TSO-negative colonies remained white.

The TSO-negative isolates were screened for known products of strain CHA0 (HCN, 2,4-diacetylphloroglucinol, pyoluteorin and pyoverdine) according to methods described earlier (Keel *et al.*, 1989, 1990; Voisard *et al.*, 1989).

The gnotobiotic system. This has been described in detail elsewhere for tobacco (Keel *et al.*, 1989). Briefly, an artificial soil was made up from pure vermiculite clay (expanded with 30% H_2O_2), quartz sand and quartz powder at (10:70:20, by wt). After moistening with 10% (w/w) H_2O, the soil was distributed into flasks, which were plugged with cotton-wool and autoclaved. The soil volume was 75 cm^3 per flask for tobacco, and 400 cm^3 per 1 litre Erlenmeyer flask for wheat. *P. fluorescens* (10^7 c.f.u. per cm^3 soil), *T. basicola* (5 × 10^3 endoconidia per cm^3 soil) for tobacco or *G. graminis* var. *tritici* grown on millet (1·25 mg per cm^3 soil) for wheat were added. After incubation for 1 week, sterile-grown tobacco plants (*Nicotiana glutinosa* L.) with four leaves or 2-d-old sterile-grown wheat seedlings (*Triticum aestivum* cv. Arina) were transplanted into the soil and supplemented with modified Knop nutrient solution (Keel *et al.*, 1989). Plants were grown in growth chambers at 18 °C (light, 12 h) and 13 °C (dark, 12 h) for wheat, and at 22 °C (light, 16 h) and 15 °C (dark, 8 h) for tobacco. After 3 weeks plants were weighed and assessed for disease severity (Stutz *et al.*, 1986; Keel *et al.*, 1989; Weller & Cook, 1983). The means of three experiments consisting of eight replicates per experiment and one tobacco plant per replicate, or three replicates per experiment and five wheat plants per replicate, respectively, were analysed statistically using the Student's *t*-test ($P = 0·05$). Each mean was compared to each other in real numbers. To lower the pH of the rhizosphere, $Ca(NO_3)_2$ of the Knop nutrient solution was substituted by 1·7 mM-$CaCl_2$ and 4·2 mM-$(NH_4)_2SO_4$ (Smiley & Cook, 1973). To obtain a lower pH of the soil, 80% of the vermiculite mixed in the soil was treated with HCl. To achieve this, 100 g expanded (H_2O_2) and ground (120 μm) vermiculite (Keel *et al.*, 1989) was suspended in 450 ml 0·44 M-HCl, stirred for 15 min and washed three times with distilled water. The pH of the artificial soil was measured according to the method of Smiley & Cook (1973): 10 g of soil was suspended in 25 ml 10 mM-$CaCl_2$, shaken for 1 h at 200 r.p.m. at room temperature and centrifuged at 4000 *g* for 5 min. For measuring the rhizosphere pH, roots were shaken in 10 mM-$CaCl_2$ to suspend the adhering soil. The pH was measured 5 min after introducing the electrode into the clear supernatant.

Reagents. These were of the highest purity grade available and were obtained from Fluka (Buchs, Switzerland) except *N*-acetyl-L-tryptophanamide, indole-3-acetaldehyde sodium bisulphite and indole-3-acetamide, which were obtained from Sigma.

Results and Discussion

IAA pathways of strain CHA0

The enzymes catalysing the initial steps of three bacterial IAA pathways (Fig. 1) were assayed in strain CHA0. Trp transaminase and TSO activities were detected (Table 1) whereas no Trp mono-oxygenase and indoleacetamide

Table 1. *Enzyme activities of cell extracts of strain CHA0*

All cultures were grown in OS minimal medium with or without 10 mM-L-Trp. Cell extracts were prepared as described in Methods. Mean values of two separate experiments did not differ by more than about ±12% of the mean value.

Culture conditions	Specific activity [mU(mg protein)$^{-1}$]	
	TSO	Trp transaminase
Exponential* − Trp	<0·2	6·2
Exponential* + Trp	<0·2	8·5
Stationary† − Trp	14·5	6·9
Stationary† + Trp	3·8	9·4

* Cells harvested in the exponential phase of growth after 16 h. The cell density was approximately 10^8 c.f.u. ml^{-1}.

† Cells harvested in the stationary phase of growth after 36 h. The cell density was approximately $1·5 \times 10^9$ c.f.u. ml^{-1}.

hydrolase activities could be measured [<0·01 mU (mg protein)$^{-1}$]; in a *P. syringae* subsp. *savastanoi* strain, used as a positive control, the latter two enzymes were readily detectable [>8 mU (mg protein)$^{-1}$]. By fractionation of a cell extract of strain CHA0 on DEAE-Sepharose, two distinct peaks of Trp transaminase activity were found (data not shown). The specific activity in both peaks was constitutive, i.e. not dependent on the presence of L-Trp in the growth medium. The substrate specificity of the two transaminase fractions was not determined; however, both also had activity toward L-Phe. TSO activity appeared only in cell extracts of stationary-phase cultures and was repressed fourfold by the addition of 10 mM-L-Trp to the culture medium (Table 1). Specific TSO activity was higher in cells of strain CHA0 grown in NYB and harvested after 36 h [approximately 20 mU (mg protein)$^{-1}$] than in cells grown in the defined OS medium (Table 1).

TSO is reported by Takai & Hayaishi (1987) to catalyse a broad spectrum of reactions *in vitro* where the side-chain of Trp and other indole derivatives, or even Trp residues in polypeptides and proteins, are oxidized. Studies by Narumiya *et al.* (1979) showed that resting cells of *P. fluorescens* (ATCC 29574) incubated with Trp produce indole-3-acetaldehyde, indoleglycolaldehyde and indoleglycoi. Each of these indolealdehydes is further oxidized or reduced to the corresponding indole acid or alcohol, respectively, by the action of an aldehyde dehydrogenase or an alcohol dehydrogenase. However, in Fig. 1 only one product of TSO, indoleacetaldehyde, is shown because this compound is a precursor of IAA. Indoleacetaldehyde dehydrogenase, which produces IAA (Fig. 1), was constitutive in strain CHA0; cell extracts of both exponential and stationary OS cultures had a specific activity of approximately 2 mU (mg protein)$^{-1}$.

Indolepyruvic acid, the intermediary product of the pathway initiated by Trp transaminase, is decomposed spontaneously to IAA and indolealdehyde, whereas indolelactic acid is formed enzymically (Kaper & Veldstra, 1958). In OS medium, IAA formation by the spontaneous breakdown of indolepyruvic acid could be measured but did not account for the bulk of IAA measured in the culture supernatant of strain CHA0 (data shown below). Therefore, an enzymic conversion of indolepyruvic acid to indoleacetaldehyde and IAA by strain CHA0 can be assumed.

Tn5 insertion mutagenesis

Since strain CHA0 has two different pathways for the synthesis of IAA, and two distinct fractions with Trp transaminase activity were detected after chromatography on DEAE-Sepharose, it was not surprising that no Tn*5* insertion mutants were found which were totally IAA-negative (data not shown). For this reason we focused on mutants lacking TSO; among 2200 kanamycin-resistant colonies isolated after Tn*5* mutagenesis, five were TSO$^-$ on malt and KB agar. Colonies of strain CHA0 and most of the putative Tn*5* insertion mutants, which were overlaid by the Trp-agar, turned tan-coloured to dark brown overnight, whereas TSO-deficient colonies remained colourless. The TSO$^-$ phenotype of the five mutants was verified by the *N*-acetyl-L-tryptophanamide enzyme assay with cell extracts. The five TSO$^-$ mutants were also screened for other extracellular products of strain CHA0. One of these mutants showed a pleiotropic deficiency in the production of 2,4-diacetylphloroglucinol, whereas four of them produced wild-type amounts of HCN, 2,4-diacetylphloroglucinol, pyoluteorin and pyoverdine and were assumed to be specifically defective in TSO formation. One representative of the latter type was named CHA750 and retained for further studies.

Role of TSO in IAA production by cells of P. fluorescens strain CHA0

IAA and several other Trp derivatives were separated by HPLC and quantified according to their fluorescence. The retention times of indole compounds were 4·8 min for Trp, 8·1 min for indoleacetamide, 10·1 min for IAA, and 10·8 min for indoleacetaldehyde and indole-ethanol, which were not separated under these conditions (Fig. 2). Indolealdehyde and indolepyruvic acid were eluted at 11·7 and 13·3 min, respectively, and detected by absorbance at 300 and 327 nm, respectively, with a detection limit of 50 ng. The detection limit for compounds detected by fluorescence was between 1 and 5 ng, and 10 ng for Trp. The determination of IAA by HPLC was verified by TLC: blue-coloured spots of the culture

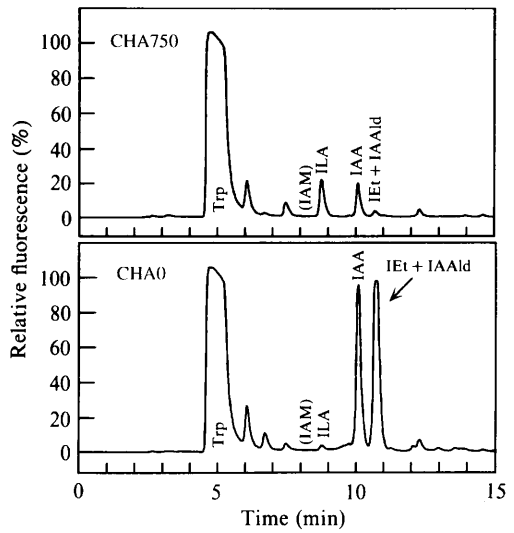

Fig. 2. HPLC separation of supernatants of resting cells of the TSO-deficient Tn*5* insertion mutant CHA750 and its parent strain CHA0. The cells were grown in NYB for 36 h and suspended in 100 mM-MES/potassium phosphate buffer (pH 5·5) containing 1 mM-L-Trp. Incubation time was 6 h. Synthetic indole compounds were used for calibration of the HPLC system and for tentative identification of fluorescent compounds produced and released by the cells. For conditions of separation, see Methods. Abbreviations: IAA, indole-3-acetic acid; IAAld, indole-3-acetaldehyde; IAM, indole-3-acetamide; IEt, indole-3-ethanol; ILA, indole-3-lactic acid; Trp, L-tryptophan.

supernatants corresponded to the R_F value (0·51–0·55) and to the colour formed by spots of synthetic IAA.

Resting cells were incubated in the presence of 1 mM-L-Trp at pH 5·5 for 6 h. The supernatant of strain CHA750 contained a reduced amount of IAA, as compared to the wild-type (Fig. 2). Moreover, the relative amounts of indolelactate excreted by strain CHA750 were elevated and those for indole-ethanol/indoleacetaldehyde reduced, suggesting that the residual amount of IAA produced by the mutant was synthesized via the indolepyruvic acid pathway (Fig. 1). In contrast, for strain CHA0, the indolepyruvic acid pathway seemed to be of minor significance compared to the TSO-catalysed formation of indoleacetaldehyde and IAA under these conditions (Fig. 1). The effect of pH on IAA production was assessed. At pH 5·5, resting cells of strain CHA750 produced up to ten times less IAA than did cells of strain CHA0. At pH 6, there was a 5-fold difference and at neutral pH the production of the mutant was 1·5-fold lower (Table 2). This suggests that the TSO pathway, at pH values below 7, accounts for most of the IAA produced by strain CHA0.

When strains CHA0 and CHA750 were grown in liquid cultures at pH 6·8 with 10 mM-L-Trp, no significant differences in IAA accumulation were measured. This can be explained by the repression of TSO when

Table 2. *IAA production of resting cells in buffers containing 1 mM-L-Trp*

Cells were harvested in the stationary phase of growth (36 h) of an NYB culture. Cell density was approximately $1·5 \times 10^9$ cells ml^{-1}. The release of IAA was measured in the supernatant at 2, 4 and 6 h, and during this period IAA production was a linear function of incubation time. The mean values and standard error of two separate experiments with three repetitions per experiment are given.

	IAA production [nM h^{-1}] at:		
Strain	pH 7·0*	pH 6·0*	pH 5·5†
CHA0	167 ± 11	322 ± 9	494 ± 18
CHA750	117 ± 6	63 ± 20	46 ± 6

* 100 mM-potassium phosphate buffer.
† 100 mM-MES/potassium phosphate buffer.

10 mM-L-Trp is present in liquid cultures (Table 1) and with the pH value of the OS medium used (pH 6·8). Both strains accumulated approximately 2, 7 and 20 μM-IAA after 24, 48 and 72 h of growth, respectively. After 18 h, cells entered the stationary growth phase. In these cultures both strains produced, in addition to IAA, readily detectable amounts of indolepyruvic acid (approximately 15 μM after 24 h), indolelactic acid (approximately 12 μM after 72 h), indoleacetaldehyde/indole-ethanol (approximately 7 μM after 72 h, calculated with indole-ethanol as standard) and indolealdehyde (approximately 1 μM after 72 h). The accumulation of these intermediary products suggests a conversion of Trp to IAA initiated by Trp transaminase (Fig. 1). Indolepyruvic acid was only detected in supernatants of bacterial cultures after 24 h. Incubation of 20 μM-indolepyruvic acid in sterile OS medium for 72 h under the same conditions as the bacterial cultures gave rise to an accumulation of approximately 2 μM-IAA and 1 μM-indolealdehyde but no indolelactate, which is in accordance with the data of Kaper & Veldstra (1958).

Traces of IAA (0·1–0·5 μM) were determined in OS cultures of strains CHA0 and CHA750 supplemented with 0·5 mM- or without L-Trp after 48 and 72 h. The amounts of IAA produced by strain CHA0 are smaller by several orders of magnitude than those synthesized by *Azospirillum brasilense* (Tien *et al.*, 1979) or by *P. syringae* subsp. *savastanoi* (Surico *et al.*, 1985) in liquid cultures containing 0·5 mM-L-Trp. Only 0·1–1·5% of the L-Trp metabolized was converted to IAA by resting cells of strain CHA0. This is probably due to the competition of Trp-catabolizing pathways other than those shown in Fig. 1, such as the conversion of L-Trp to L-kynurenine and anthranilic acid. This pathway is initiated by Trp-2,3-dioxygenase and has been described for other *P. fluorescens* strains (Ishimura, 1970; Narumiya *et al.*,

1979). There is circumstantial evidence that this pathway occurs in strain CHA0. Strain CHA0 was able to grow in OS medium with L-Trp as the sole C source, and in the supernatant of OS cultures, supplemented with 10 mM-L-Trp, a compound co-eluting with authentic anthranilic acid was detected by HPLC at 327 nm, with a retention time of 6·3 min. The UV spectrum of this fraction was identical to that of synthetic anthranilic acid. The kynurenine pathway probably competes with the IAA biosynthetic pathways for Trp. When culture media containing glucose as a C source were supplemented with 10 mM-L-Trp, only part of this amount of Trp was metabolized during cell growth and some Trp was still available for IAA synthesis.

Strain CHA104, which is auxotrophic for Trp, was able to grow on roots of tobacco and wheat plants to the same extent as did strain CHA0 (data not shown). This provides evidence that Trp was present in root exudates.

Influence of the TSO mutation on disease suppression

Protection of tobacco from black root rot and of wheat from take-all disease by strains CHA0 and CHA750 was assessed in gnotobiotic systems containing artificial, vermiculite or acidified vermiculite soil. Fresh weight of plants grown without micro-organisms was taken as 100%. Plants grown in the presence of the pathogen alone weighed 21% in the case of tobacco and 50% in the case of wheat. Tobacco plants protected by strains CHA0 and CHA750 weighed 72 and 76%, respectively, and wheat plants 88 and 90%, respectively. Both strains also gave the same protection in terms of disease severity (data not shown). The rhizosphere pH was approximately 6·9–7·0 in the soil containing untreated vermiculite, and 5·9–6·1 in acidified vermiculite. We would like to point out that the pH was measured in the rhizosphere with some soil still adhering to the roots and it is likely that the pH at the root surface was somewhat lower (Smiley & Cook, 1973). When *P. fluorescens* is in the stationary growth phase and at pH values of $\leqslant 6{\cdot}0$, the contribution of TSO to IAA production is important (Table 2). However, the fact that the TSO^- mutant CHA750 gave normal disease suppression at pH ~ 7 as well as pH ~ 6 leads us to conclude that the TSO pathway does not have an important role in disease suppression.

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