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Biosynthesis of Auxin by the Gram-Positive Phytopathogen *Rhodococcus fascians* Is Controlled by Compounds Specific to Infected Plant Tissues

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The role and metabolism of indole-3-acetic acid in gram-negative bacteria is well documented, but little is known about indole-3-acetic acid biosynthesis and regulation in gram-positive bacteria. The phytopathogen *Rhodococcus fascians*, a gram-positive organism, incites diverse developmental alterations, such as leafy galls, on a wide range of plants. Phenotypic analysis of a leafy gall suggests that auxin may play an important role in the development of the symptoms. We show here for the first time that *R. fascians* produces and secretes the auxin indole-3-acetic acid. Interestingly, whereas noninfected-tobacco extracts have no effect, indole-3-acetic acid synthesis is highly induced in the presence of infected-tobacco extracts when tryptophan is not limiting. Indole-3-acetic acid production by a plasmid-free strain shows that the biosynthetic genes are located on the bacterial chromosome, although plasmid-encoded genes contribute to the kinetics and regulation of indole-3-acetic acid biosynthesis. The indole-3-acetic acid intermediates present in bacterial cells and secreted into the growth media show that the main biosynthetic route used by *R. fascians* is the indole-3-pyruvic acid pathway with a possible rate-limiting role for indole-3-ethanol. The relationship between indole-3-acetic acid production and the symptoms induced by *R. fascians* is discussed.

The phytopathogenic actinomycete *Rhodococcus fascians* induces the development of a wide range of malformations on its numerous monocotyledonous and dicotyledonous host plants: leaf deformation, growth inhibition, witches' brooms, fasciations, and leafy galls (reference 23 and references therein). The leafy-gall structure, considered an extreme form of apical dominance (59), is the most severe outcome of the *R. fascians*-plant interaction. Consisting of small misshapen leaves and numerous buds that are inhibited for further outgrowth, the leafy gall is believed to result from the alteration of the endogenous hormone balance of the host plant (23). Leafy-gall features, such as leaf wrinkling, shoot multiplication, and delayed senescence, are typical cytokinin effects. In this respect, *R. fascians* pathogenicity has been linked to a linear plasmid (pFiD188) that carries different virulence genes. On pFiD188 an isopentenyl transferase (*ipt*) homologue is located that is essential for symptom development and has been found in all virulent strains examined thus far (14, 15, 53). Moreover, it is known that *R. fascians* can produce at least 11 different cytokinins in vitro (2, 18, 24, 28, 48, 51). In *R. fascians*, cytokinins probably originate from two sources, either release from tRNA (36, 38) and/or condensation of isopentenyl pyrophosphate and 5′-AMP (14). Nevertheless, hitherto no clear correlation between known cytokinins and symptom development could be established (16, 18), suggesting that new cytokinin-like molecules could be involved in the formation of the leafy gall (23).

Formation of a leafy gall cannot be attributed solely to the action of cytokinin. Some leafy-gall characteristics, such as vascular tissue differentiation, cell enlargement, and inhibition of bud outgrowth, are known as typical auxin effects. Moreover, other phenotypes induced by *R. fascians*, such as cell swelling and lateral root initiation (59), can also be attributed to auxin. Although auxin production is reported for several plant-associated bacteria (42), only few data are available on the possible involvement of auxin during *R. fascians*-plant interaction. Although it has been suggested that the production of cytokinins and the simultaneous degradation of auxins by *R. fascians* could be at the origin of symptom development (3), elevated levels of the auxin indole-3-acetic acid (IAA) were detected in infected plant tissues compared to noninfected tissues (16, 59), suggesting that *R. fascians* synthesizes IAA and/or induces its production by the plant.

The production of IAA and its main intermediates by *R. fascians* is described here for the first time. The production of IAA by the highly virulent strain D188 and the nonvirulent strain D188-5 was compared to assess the importance of IAA secretion in symptom development. Furthermore, the effect of the natural environment of the bacteria on the production of IAA and IAA intermediates was explored. We show that *R. fascians* produces IAA mainly through the indole-3-pyruvic acid (IPyA) pathway, and we discuss the importance of IAA production in relation to the capacity of *R. fascians* to colonize plant tissues and/or to induce symptoms.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *R. fascians* strains D188 and D188-5 are, respectively, a highly virulent strain and a nonvirulent plasmid-free strain (17). Both strains were grown in liquid YEB medium (37) and cultures were

incubated at 28°C with agitation at 130 rpm (shaker Innova 2100; New Brunswick) until an optical density at 600 nm of 2 (typically, 48 h). Cells were harvested by centrifugation (5,000 × *g*, 5 min), washed in minimal medium (JM medium; 3.875 g of Gamborg's B5 salts/liter, 500 mg of morpholineethanesulfonic acid/liter, 250 mg of NH_4NO_3/liter, 0.001% thiamine-HCl; pH 5.5), and diluted 4.25-fold in JM medium. When required, tryptophan (TRP), indole-3-ethanol (IEt), indole-3-acetamide (IAM), or tryptamine (TRA) was supplied at a concentration of 20 mg/liter. When appropriate, 23.5 μl of an aqueous extract of either leafy galls (LG extract) or noninfected plant tissues (NIP extract) was added per milliliter of JM medium. All proper controls, including medium plus LG extract, medium plus NIP extract, medium plus LG extract plus TRP, medium plus NIP extract plus TRP, medium plus TRP, medium plus TRA, or medium plus IEt, were included in the experiments. In each control, the IAA levels were significantly lower than in the presence of the bacteria (data not shown). For time course experiments the samples were harvested for extraction of IAA and precursors, and for CFU measurements. The numbers of CFU were calculated by plating serial dilutions (10^{-8}, 10^{-9}, and 10^{-10}) of the samples on solid YEB medium. Plates were incubated at 28°C until single colonies could be counted to determine the number of CFU per milliliter for each bacterial culture. The cultures were centrifuged (5,000 × *g* for 5 min); bacterial cells and supernatants were separately stored at −20°C until indole analysis.

Preparation of LG and NIP extracts. *Nicotiana tabacum* cv. Havana seeds were aseptically germinated and grown (24°C, 16/8-h photoperiod at 70 μmol photons/m^2/s) on solid half-strength MS medium (39). After 4 weeks, the tobacco plants were decapitated and infected with a drop (~5 μl, optical density at 600 nm of 2) of exponentially growing *R. fascians* D188 cells (growth in YEB for 24 h) or mock inoculated with an equivalent volume of YEB medium. Eight-week-old leafy galls or mock-inoculated plants were harvested and extensively crushed in water (ca. 2 g of plant tissues for 10 ml of sterile water) and filtered to remove tissue debris. The capacity of the LG extract to induce *R. fascians* virulence gene expression was evaluated by assessing the expression of the *att* locus as described previously (32). The levels of *att*H expression in JM medium, with or without extracts of noninfected plant, were low as evaluated by measuring the β-glucuronidase activity (data not shown). However, when LG extract was added to the medium, *att*H expression was strongly induced (data not shown), confirming that the LG extract contains compounds that trigger virulence-associated gene expression as described previously (14, 32).

Extraction of IAA and precursors from liquid culture medium. The supernatants were mixed with an equal volume of 0.1 N HCl and the internal standards [$^{13}C_6$]IAA (150 ng, [phenyl-$^{13}C_6$]indole-3-acetic acid; Cambridge Isotope Laboratories, Andover, Mass.), [$^{13}C_6$]IAM (10 ng, synthesis from $^{13}C_6$-IAA), [$^{15}N_1$]IEt (100 ng, synthesis from indole[$^{15}N_1$]TRP; Cambridge Isotope Laboratories), [$^{13}C_1$]indole-3-acetonitrile ([$^{13}C_1$]IAN; 100 ng, a gift from N. Ilic), d5-TRP (100 ng of L-TRP-2′,4′,5′,6′,7′-d5; CDN Isotopes, Pointe-Claire, Quebec, Canada), and [^{15}N]AA (100 ng; Cambridge Isotope Laboratories) were added. Acidified samples were applied to a C_{18} cartridge (Bond-Elut 500 mg; Varian, Inc., Harbor City, Calif.) equilibrated with 50 mM HCl. Samples were eluted with 5 ml of acetonitrile. After vacuum evaporation (RVC2-25; Christ, Osterode am Harz, Germany), the residue was dissolved in 100% acidified methanol and methylated with diazomethane prior to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis performed as described previously (45). Although methylation is not an absolute prerequisite for analysis of indole compounds, the samples containing all indole intermediates were methylated before analysis to improve the detection limit of the carboxylic indoles and to prevent degradation of IPyA (45).

LC ES(+)-MS/MS analysis. Micro LC was performed on a Prodigi 5 μm OD83 100 to 150 mm by 1 mm (Phenomenex, Torrance, Calif.) column. The total sample volume injected was 25 μl. The mobile phase consisted of the following gradient system: 1 min, sample loading at 10/90 (vol/vol) methanol-ammonium oxaloacetate (MeOH-NH_4OAc; 0.01 M; pH 7); from 1 to 5 min, a linear gradient from 10/90 to 95/5 (vol/vol) MeOH-NH_4OAc (0.01 M; pH 7); from 5 to 7.5 min, isocratic at 95/5 MeOH-NH_4OAc (0.01 M); and from 7.5 to 11 min, isocratic equilibration at start conditions at 10/90 (vol/vol) MeOH-NH_4OAc (0.01 M; pH 7). A constant flow rate of 60 μl/min was achieved by using a Kontron BIO-TEK P522 binary gradient pump (Kontron Instruments, Milan, Italy) with mixing at high pressure. The effluent was directly introduced at a flow rate of 60 μl/min into the MS source. The LC system was linked to a Quattro II mass spectrometer (Micromass, Ltd., Manchester, United Kingdom) equipped with an electrospray (ES) interface and Z-spray (Micromass). The source temperature was 80°C, the nebulizing gas flow was 20 liters/h, the drying gas flow was 400 liters/h, and the capillary voltage was +3.5 kV. The cone voltage depended on the appropriate compound (45, 46). Collision activated dissociation of the protonated molecular ion ([MH]$^+$) was obtained by using argon as a collision gas at the appropriate compound-specific collision energy, which ranged between 10 and 20 eV (46), and a PAR of 4.10 to 3 mbar. Quantification was done by multiple reactant monitoring of the [MH]$^+$ ion (dwell time, 0.05 s; interchannel delay, 0.01 s; span, 0 atomic mass unit) and the appropriate product ion. All indole compounds present in one sample were analyzed simultaneously during a single LC-MS/MS run. All mass spectra were background subtracted and smoothed once. All data were processed by using Masslynx 3.5 software.

RESULTS

The virulent *R. fascians* strain D188 produces IAA independently from the TRP supply. The production of IAA by the highly virulent *R. fascians* strain D188 was assessed during growth in a minimal medium. At regular time intervals over a period of 106 h, IAA levels present in bacterial cells and secreted into the culture media were measured. Because of the selectivity of multiple reactant monitoring MS/MS, using a unique diagnostic transition for each compound analyzed based on tandem mass spectrometric analysis, the MS/MS data are not only used for quantification but are also a reliable identification criterion for the compounds analyzed (45). We report only on the IAA levels secreted by *R. fascians* in the culture media, since in the bacterial cells 200-fold-lower levels were detected (data not shown), although the kinetic profiles for both samples were comparable. IAA concentrations increased linearly in time, reaching 285 ± 23 pmol/ml/10^{11} CFU at the end of the experiment (Fig. 1A). Only background traces of IAA were detected in the bacterium-free media (between 1 and 5 pmol/ml), indicating that *R. fascians* strain D188 produces IAA under the growth conditions tested. Because L-TRP, considered to be the main IAA biosynthetic precursor (42), could be limiting for IAA biosynthesis, *R. fascians* D188 was also grown in a minimal medium supplemented with TRP (20 mg/liter). Under these conditions IAA concentrations also increased linearly with time, and the level of secreted IAA was comparable to that measured upon growth in TRP-free medium (Fig. 1A). As shown in Fig. 1B, the growth of strain D188 in the minimal medium is stable during the time course of the experiment. Addition of TRP to the medium had no major effect on the growth of *R. fascians* D188.

An LG extract induces IAA production by *R. fascians* strain D188 upon TRP supply. It has been shown that LG extracts contain specific compounds that are not found in noninfected plant tissues and that trigger specific responses in *R. fascians*, such as induction of the expression of virulence genes (14, 32, 54). Therefore, the level and kinetics of IAA production by *R. fascians* were investigated when the bacteria were grown in a medium supplemented with LG extract. Under these conditions, IAA concentrations increased linearly and reached levels comparable to those detected in LG extract-free cultures (Fig. 2A). However, when TRP was added to the minimal medium supplemented with LG extract, IAA secretion by strain D188 significantly increased two- to sixfold (Fig. 2A). IAA levels reached a maximum of 640 ± 60 pmol/ml/10^{11} CFU after 58 h of incubation and then slightly decreased until the end of the experiment, reaching a level of 508 ± 36 pmol/ml/10^{11} CFU. In the presence of an NIP extract and TRP, IAA secretion by strain D188 was comparable to that of bacteria grown in LG extract-supplemented medium in the absence of exogenous TRP (Fig. 2A). The induction of IAA synthesis in vitro was not the result of a supply of IAA or its precursors, nor of a stimulation of the bacterial growth by the LG extracts itself (Fig.

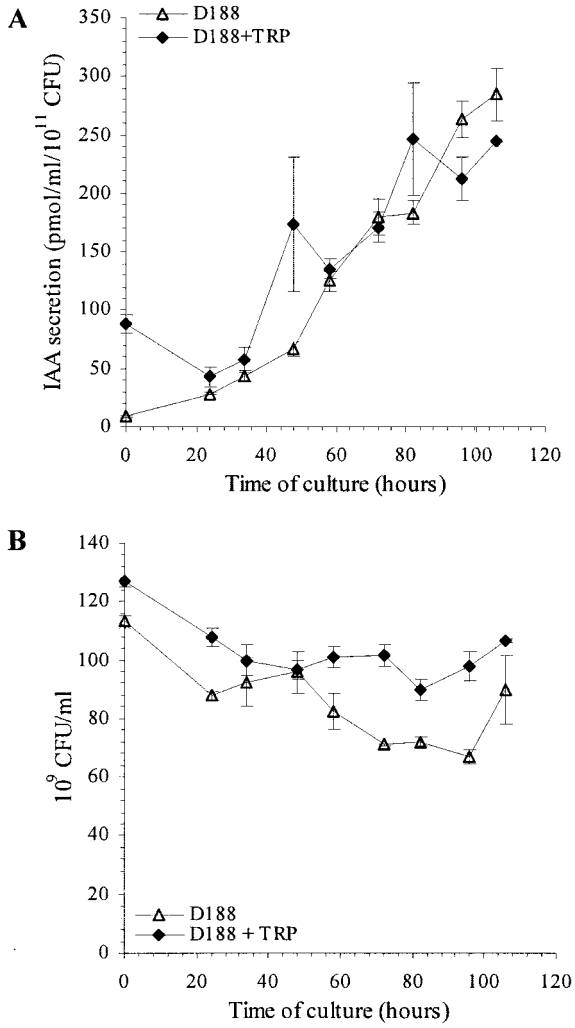


FIG. 1. Kinetics of IAA secretion by (A) and growth of (B) *R. fascians* D188. IAA levels and growth were measured in three independent experiments. Error bars represent the standard errors.

2B). This indicates that leafy-gall-specific compounds have a major effect on IAA secretion by *R. fascians*.

The nonvirulent, plasmid-free *R. fascians* strain D188-5 has a delayed IAA production. IAA production by the plasmid-free strain D188-5 was also evaluated. This nonvirulent strain was incubated in TRP-supplemented minimal medium, and IAA levels were measured at regular time intervals over a period of 106 h. IAA levels remained low and constant during the first 58 h of culture and then increased linearly, reaching a maximum level after 84 h (Fig. 3A) that was comparable to the level produced by strain D188. In the presence of both TRP and LG extract, IAA levels remained constant (between 70 and 90 pmol/ml/10^{11} CFU) during the first 58 h of culture and then increased rapidly to reach levels comparable to those measured for strain D188 cultured under similar conditions. These data indicate that the IAA biosynthetic genes are located on the bacterial chromosome. The observed delay in IAA production by strain D188-5 suggests that a plasmid-encoded locus contributes to the regulation of IAA biosynthesis in *R. fascians*. As shown in Fig. 3B, addition of LG extract had no major effect on the growth of strain D188-5.

Kinetics of IAA intermediates during culture growth of virulent and nonvirulent *R. fascians* strains. The secretions of known IAA intermediates by the two *R. fascians* strains were compared to pinpoint an eventual limiting step in IAA biosynthesis and to investigate the biosynthetic pathways involved. Because the levels of several IAA-intermediates were extremely low or under the detection limit upon growth in TRP-free medium (data not shown), analyses were performed on TRP-supplemented bacterial cultures. IAA conjugates were below the detection limit under all conditions tested (i.e., both strains grown in TRP-supplemented medium with or without addition of the LG extract) both in supernatants and bacterial

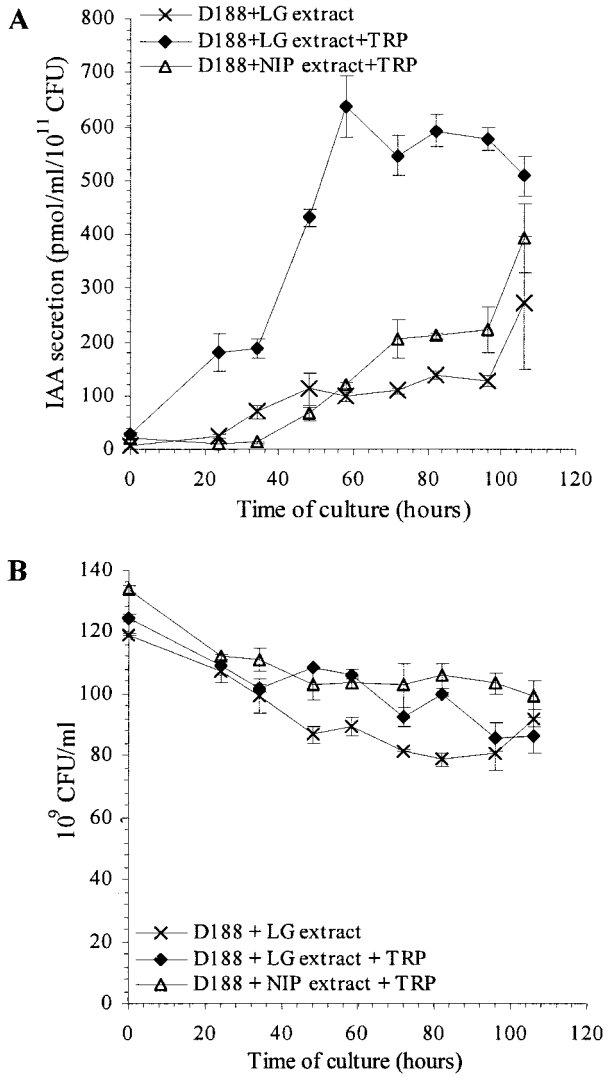


FIG. 2. Kinetics of IAA secretion by (A) and growth of (B) *R. fascians* D188 upon addition of plant extracts. Bacteria were grown in the minimal medium supplemented with LG extract (×) and in the TRP-supplemented medium after the addition of either LG extract (◆) or NIP tissue extract (△). IAA levels and growth were measured in three independent experiments. Error bars represent the standard errors.

cells (data not shown), indicating that there is no significant production or breakdown of IAA conjugates. Similarly, the IAA intermediates (see Fig. 6) TRA, IAN, IAM, and indole-3-acetaldehyde were present in very low concentrations or were under the detection limit (data not shown).

The main indolic compounds secreted to the medium upon TRP supplementation were the same for both strains, and all were intermediates of the IPyA pathway: IPyA, indole-3-aldehyde (IAld), indole-3-lactic acid (ILA), and IEt. IPyA, IAld, and ILA concentrations increased with time concomitantly with the increase in IAA levels (Fig. 4A to C). In contrast, IEt levels transiently increased during the first 48 h of culture and then decreased until the end of the experiment (Fig. 4D). Finally, the levels of TRP dropped rapidly within the first 40 h of culture (Fig. 4E), along with the rapid increase in IPyA concentrations (Fig. 4A).

The levels of IAA intermediates were also investigated in both strains upon addition of TRP and LG extract (Fig. 4F to J). IPyA levels increased concomitantly with the decrease in TRP level (Fig. 4F and J, respectively). When compared to the LG extract-free cultures, slightly higher IAld levels were detected in the media of both strains (Fig. 4G), whereas ILA production decreased in both strains (Fig. 4H). Particularly in the presence of LG extract, significantly higher amounts of IEt were measured for both strains (Fig. 4I). In the culture medium of strain D188, the IEt levels decreased rapidly after 24 h. For strain D188-5 the IEt level remained high until 58 h of culture, after which it dropped rapidly. The observed rapid decrease of IEt in both strains coincided with an increase in IAA levels (see Fig. 3A), suggesting that IEt is converted into IAA. A similar kinetic profile of IEt production was observed in the absence of LG extract, although the maximal production level was significantly lower (Fig. 4D). The increased concentrations of indolic compounds did not result from the addition of the LG extract, since only low levels of IAA (2.1 ± 0.5 pmol/ml), IAld (145 ± 13 pmol/ml), anthranilic acid (110 ± 10 pmol/ml), and TRP (4,100 ± 100 pmol/ml) could be detected in bacterium-free, LG extract-supplemented media. The concentrations of all of the other IAA intermediates in this medium were under detection limits (data not shown).

The overall data from the kinetic studies of the IAA intermediates indicate that in *R. fascians* strain D188 the IAA is mainly synthesized via the IPyA pathway under the conditions tested. Nevertheless, the capacity of *R. fascians* to use other IAA biosynthetic pathways was assessed by feeding experiments with two IAA precursors that were detected in low concentrations in *R. fascians* cultures: IAM and TRA. IAM was clearly taken up by the bacterial cells (data not shown); however, this feeding did not result in an increase of the IAA levels (data not shown), indicating that the IAM pathway is probably not used by *R. fascians* in the applied growth conditions. When *R. fascians* D188 was incubated in a minimal medium supplemented with LG extract and TRA (20 mg/ml), IAA was no longer secreted although TRA had no effect on bacterial growth (data not shown).

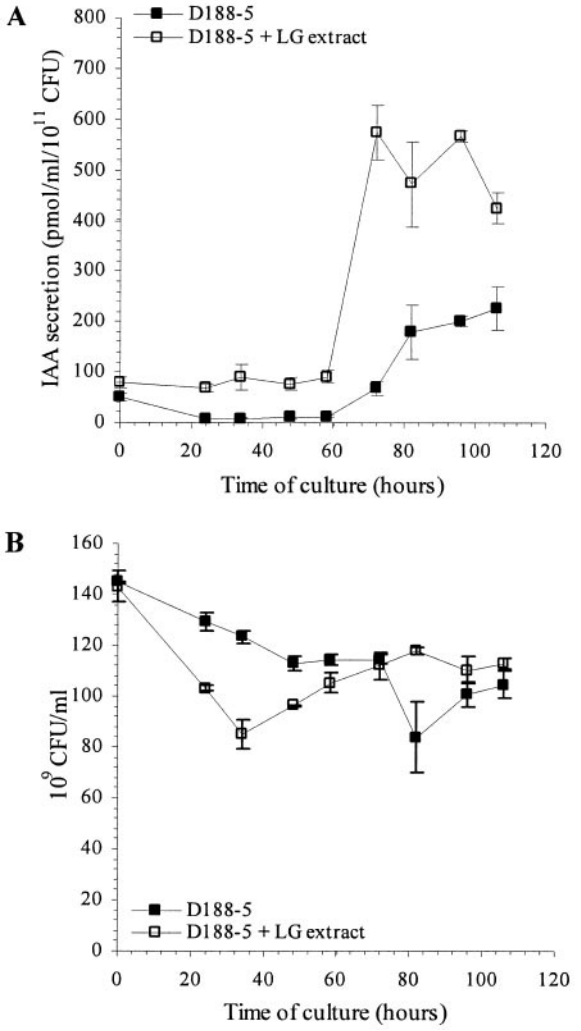
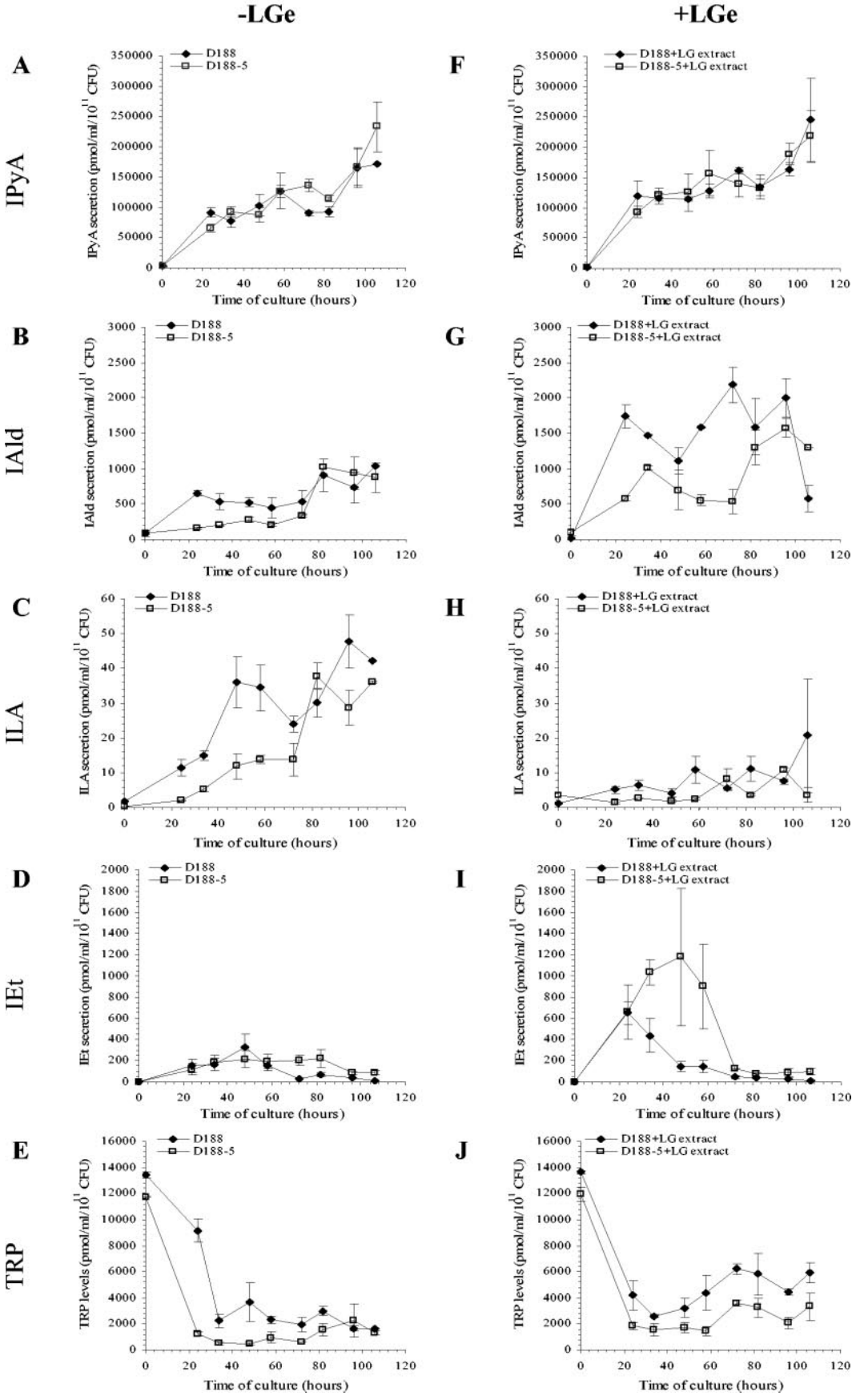


FIG. 3. Kinetics of IAA production by (A) and growth of (B) *R. fascians* D188-5. Bacteria were grown in TRP-supplemented medium with (□) or without (■) LG-extract. IAA levels and growth were measured in three independent experiments. Error bars represent the standard errors.

***R. fascians* converts IEt into IAA.** Comparison of IAA and IEt kinetic studies (see Fig. 2A, 3A, and 4D and I) strongly suggested that IEt was converted into IAA. To test this hypothesis, we conducted feeding experiments in which both strains were incubated in IEt-supplemented TRP-free medium. Very high levels of IAA were reached (26,800 ± 1,500 pmol/ml/10^{11} CFU) within 24 h of incubation (Fig. 5), suggesting that IEt is rapidly converted into IAA and that the production of IEt could constitute a limiting step in IAA biosyn-

FIG. 4. Kinetics of IAA intermediate secretion by *R. fascians* strains D188 (♦) and D188-5 (□). Bacteria were grown in TRP-supplemented medium in the absence (A to E) or in the presence (F to J) of LG extract (LGe). (A and F) IPyA levels; (B and G) IAld levels; (C and H) ILA levels; (D and I) IEt levels; (E and J) TRP levels. IAA levels were measured in three independent experiments. Error bars represent the standard errors. The corresponding IAA data of these samples are presented in Fig. 2 and 3.


-LGe
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D188+LG extract
D188-5+LG extract
IPyA secretion (pmol/ml/10^11 CFU)
IAld secretion (pmol/ml/10^11 CFU)
ILA secretion (pmol/ml/10^11 CFU)
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TRP levels (pmol/ml/10^11 CFU)
Time of culture (hours)

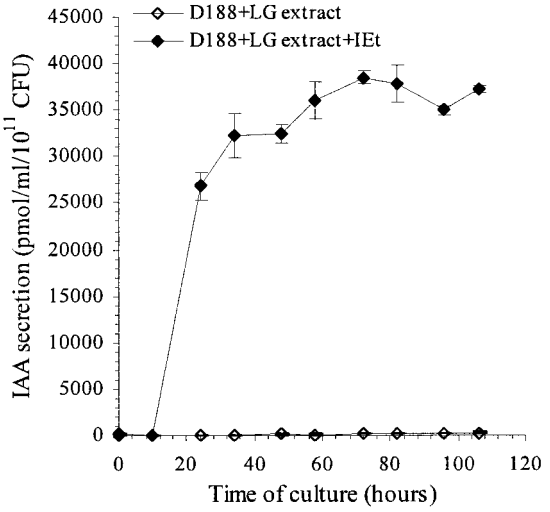

FIG. 5. Conversion of IEt into IAA by *R. fascians* strain D188. The kinetics of IAA production by *R. fascians* in medium supplemented with LG extract (◇) and with both LG extract and IEt (20 mg/liter) (◆) are indicated. IAA levels were measured over a 106-h growth period in three independent experiments. Error bars represent the standard errors.

thesis by *R. fascians*. Both strains were able to convert IEt into IAA irrespective of the presence of LG extract. In all cases, the IAA levels detected in culture medium were ca. 100 times higher than without IEt (data not shown). The conversion of IEt into IAA could not be attributed to a nonspecific degradation of IEt since only very low levels of IAA were detected in the bacterium-free medium containing both IEt and the LG extract (data not shown).

DISCUSSION

The shooty phenotype induced by *R. fascians* on its host plants directed earlier studies toward the synthesis of cytokinins by this bacterium (2, 18, 24, 28, 48, 51). Nevertheless, careful examination of the various symptoms resulting from *R. fascians* infection revealed that auxin could also play a role during the interaction with the plant (16, 59). The involvement of auxin had only been considered at the level of degradation (3, 27, 50), and possible synthesis of IAA by *R. fascians* had not been evaluated.

We show here for the first time that *R. fascians* is able to produce and secrete significant amounts of IAA. The amount of IAA secreted by these bacteria is higher than or comparable to that secreted by the plant pathogens *Agrobacterium rhizogenes* and *Xanthomonas campestris*, respectively, and comparable to that secreted by the plant-growth-promoting bacterium *Azospirillum irakense* (E. Prinsen, unpublished results). IAA synthesis is a common feature of plant-pathogenic and plant-growth-promoting bacteria (see, for example, reference 42) and is not restricted to gram-negative bacteria (21). Generally, IAA production increases after the addition of TRP to the growth medium (20, 26, 29). In the case of *R. fascians*, the addition of a minimal amount of TRP to the growth medium had no significant effect on IAA production. Interestingly, the amount of IAA secreted by *R. fascians* in the culture medium was highly increased upon the addition of infected-plant extracts only when TRP was supplied. These data suggest that a TRP threshold concentration and infected-plant-tissue compound(s) are required simultaneously. Although TRP synthesis is a high energy-consuming process (1, 13) and the TRP level in plant tissues is low (47), these data support the idea that TRP-dependent IAA synthesis rises only when *R. fascians* grows in appropriate symptomatic-tissue-specific conditions that are mimicked in the in vitro growth condition used in the present study. Possibly, one or more IAA biosynthetic genes are regulated by compound(s) synthesized during the establishment of the leafy gall. In *R. fascians*, it is known that the expression of virulence-associated genes, such as *fas* and *att*, is induced in vitro by an aqueous LG extract but not by NIP extracts (14, 32, 54). On the other hand, expression of the chromosomal *vic* locus is induced by both extracts (60). Upon addition of NIP extracts, IAA secretion did not increase, suggesting that leafy-gall-specific compounds are responsible for the observed enhancement of IAA production. Previous studies have indeed shown that the composition of amino acids and phenolic compounds differs between *R. fascians*-infected plant tissues and uninfected plants (19, 58). Our data and previous reports also indicate that bacterial gene expression and physiology are modified in the presence of LG extracts. Nevertheless, the structure of the activating compounds in the LG extract is currently unknown. Furthermore, whether the compounds inducing virulence-associated gene expression in *R. fascians* are those involved in the stimulation of IAA production remains to be determined. In other bacteria that synthesize IAA, environmental signals, such as particular host specific flavonoids (44, 56), compounds present in leaf extracts (12), the plant surface environment (8), osmotic stress (7), or auxins (57), are known to affect IAA production or IAA biosynthetic gene expression (8).

Based on the IAA precursor analyses, the IPyA pathway is likely the only IAA biosynthetic route used by *R. fascians* in the growth conditions tested. Indeed, the main IAA intermediates detected belong to the IPyA pathway (Fig. 6), and precursors of all other IAA biosynthetic pathways were detected in concentration below or near the detection limits. The eventual contribution of other pathways, such as the TRA and IAM pathways frequently found in bacteria (42), to the IAA biosynthesis in *R. fascians* was tested by feeding experiments, but none resulted in a clear production of IAA. The TRP side chain oxidation pathway shares IAA precursors with the IPyA pathway, but its role in IAA biosynthesis in *R. fascians*, as is the case in *Pseudomonas fluorescens* (40, 41), is doubtful based on the kinetics of ILA and IPyA. Comparison of IAA production by strain D188 and strain D188-5, a plasmid-free strain, indicates that the IAA biosynthetic genes are located on the bacterial chromosome. This is consistent with the chromosomal location of the genes encoding the IPyA pathway in other bacteria (42).

Irrespective of the presence of LG extract, strain D188 started to produce IAA earlier than did strain D188-5. Interestingly, whatever the case, the progressive increase in IAA levels coincided with the decrease in IEt levels in both strains. The capacity of *R. fascians* to use IEt as a precursor to synthesize IAA was further confirmed by feeding experiments. The regulatory role of IEt in IAA biosynthesis is strengthened

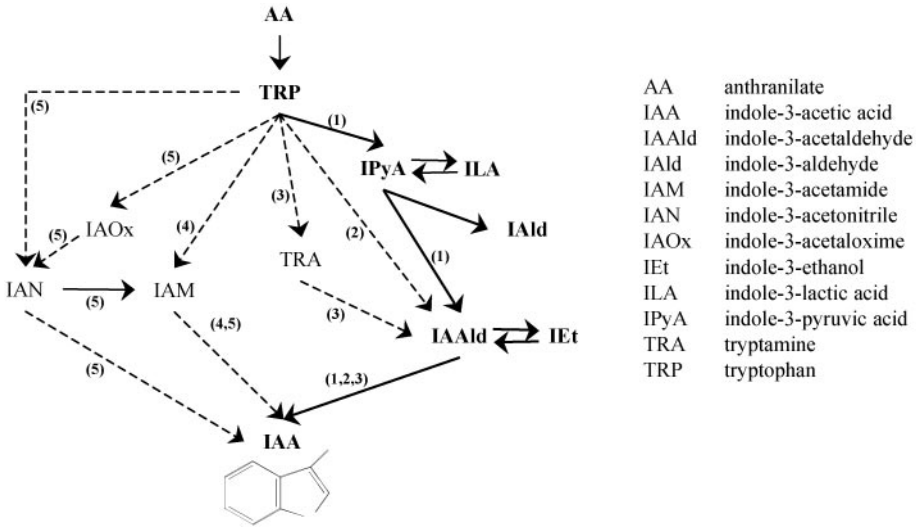

FIG. 6. Schematic representation of IAA biosynthetic pathways in *R. fascians* and other plant-associated bacteria. Numbered arrows: 1, the IPyA pathway; 2, the tryptophan side chain oxidation pathway; 3, the TRA pathway; 4, the IAM pathway; 5, the IAN pathway. The IAA pathway in *R. fascians* is indicated by continuous lines. (The diagram follows the example set in references 11 and 42).

by the fact that addition of TRP does not result in accumulation of IAA, as was shown for all IAA intermediates in the IPyA pathway. Conversion of IEt into IAA was also observed in *Streptomyces* spp. (35) and in *Rhizobium lupini* (21). This step in the IPyA pathway is indeed considered a regulatory reaction for IAA synthesis in bacteria (5, 21) and also in plants (55). Since strain D188-5 lacks the virulence-associated linear plasmid pFiD188 and the cadmium resistance-associated plasmid pD188 (17), plasmid-encoded proteins are likely contributing to the production of IAA through the conversion of IEt. Indeed, it has already been shown that different virulence-associated loci require plasmid-encoded factors to be optimally expressed (54, 60). Another possibility is that one of the plasmids carries a gene involved in the conversion of IEt into IAA, explaining the rapid production of IAA in strain D188. Nevertheless, since IAA production from IEt also occurs in strain D188-5, other signals must be involved in the regulation of the IAA biosynthetic genes. In this context, it has recently been shown that stress-specific "σ factors" are involved in the synthesis of IAA by *Pseudomonas putida* GR12-2 (43), *Rhizobium* sp. strain NGR234 (56), and *Azospirillum brasilense* (30).

Based on all currently available data, a working hypothesis is proposed in which the basal IAA production contributes to the colonization efficiency and to the growth and survival of *R. fascians* on its host plants. Once the development of the symptoms induced by *R. fascians* is initiated, bacterium-derived IAA synthesis is stimulated by compounds produced in infected plant tissues. This boost of auxin, which is comparable to 10 times the concentration per gram (fresh weight) present in noninfected plant tissues, participates in the disruption of the hormonal balance of the host. The latter is indicated by the high IAA levels detected in infected plants (16, 59), and its physiological relevance is illustrated by some of the phenotypic features of leafy galls, such as the inhibition of bud outgrowth. This working hypothesis is supported by several reports on different plant-associated bacteria that show the importance of bacterial IAA release for full development of the induced symptoms or for epiphytic fitness. Indeed, in *Pantoea agglomerans* (*Erwinia herbicola* pv. *gypsophilae*), the IPyA pathway has been associated with epiphytic fitness of the bacteria on the host plants, while the IAM pathway has been linked to the development of galls induced by this bacterium (33, 34). Likewise, the importance of IPyA-derived IAA in the epiphytic fitness of nonpathogenic *Erwinia herbicola* strains was exemplified by the less efficient plant colonization of IAA mutants compared to parental strains (6). Also, *Pseudomonas syringae* pv. *savastanoi* requires IAA for colonization and survival on its host and for symptom development (9, 10, 25, 52). Proposed roles for bacterial IAA synthesis include the detoxification of TRP analogues present on host plant surfaces (4, 31), the stimulation of the release of plant metabolites (5, 22, 30), the downregulation of plant defense (61), and/or the inhibition of the hypersensitive response of infected plants (49).

In conclusion, the work presented here demonstrates that *R. fascians*, a teratogenic phytopathogen generally associated merely with cytokinins, is also able to produce the auxin IAA through the IPyA pathway. Identification of this biosynthetic route is a prerequisite for the isolation of the genes involved and for the molecular analysis of this pathway in gram-positive bacteria. It is the first important step toward understanding the role of auxin in the interaction of *R. fascians* with plants.

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