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Tryptophan-Dependent Production of Indole-3-Acetic Acid (IAA) Affects Level of Plant Growth Promotion by *Bacillus amyloliquefaciens* FZB42

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Phytohormone-like acting compounds previously have been suggested to be involved in the phytostimulatory action exerted by the plant-beneficial rhizobacterium *Bacillus amyloliquefaciens* FZB42. Analyses by high-performance liquid chromatography and gas chromatography-mass spectrometry performed with culture filtrates of FZB42 demonstrated the presence of indole-3-acetic acid (IAA), corroborating it as one of the pivotal plant-growth-promoting substances produced by this bacterium. In the presence of 5 mM tryptophan, a fivefold increase in IAA secretion was registered. In addition, in the *trp* auxotrophic strains E101 (*ΔtrpBA*) and E102 (*ΔtrpED*), and in two other strains bearing knockout mutations in genes probably involved in IAA metabolism, E103 (*ΔysnE*, putative IAA transacetylase) and E105 (*ΔyhcX*, putative nitrilase), the concentration of IAA in the culture filtrates was diminished. Three of these mutant strains were less efficient in promoting plant growth, indicating that the Trp-dependent synthesis of auxins and plant growth promotion are functionally related in *B. amyloliquefaciens*.

Enhancement of plant growth by root-colonizing *Bacillus* and *Paenibacillus* strains is well documented (Kloepper et al. 2004; Timmusk and Wagner 1999; Yao et al. 2006). Moreover, plant-growth-promoting rhizobacteria (PGPR) of the *Bacillus* group offer a biological solution to the formulation problem due to their ability to form heat- and desiccation-resistant spores (Emmert and Handelsman 1999). However, very little is known about the basic molecular mechanisms responsible for beneficial action of the bacilli group of PGPR, preventing development of optimal application strategies for available formulations in agriculture and horticulture. It is very likely that plant growth promotion by rhizosphere bacilli results from the combined action of several factors. The volatiles 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol, released by *Bacillus subtilis* and *B. amyloliquefaciens*, trigger enhanced plant growth (Ryu et al. 2003). In previous studies, we showed that biofertilization exerted by extracellular bacterial phytase under conditions of phosphate limitation and in the presence of phytate can contribute to the plant-growth-promoting activity of *B. amyloliquefaciens* FZB strains (Idriss et al. 2002; Makarewicz et al. 2006). Suppression of the competitive plant-pathogenic microflora within the rhizosphere by secreted antifungal and

antibacterial lipopeptides and polyketides might be important for promotion of plant growth by FZB42 (Chen et al. 2006; Koumoutsis et al. 2004). In addition, *B. amyloliquefaciens* FZB42 enhanced development of plant resistance against competitive pathogenic fungi and bacteria (Kilian et al. 2000).

It also is likely that plant-growth-promoting effects exerted by some plant-beneficial bacteria are due to the bacterial production of plant hormones such as indole-3-acetic acid (IAA), cytokinins, and gibberellins (Bloembergen and Lugtenberg 2001; Bottini et al. 2004). IAA was detected in 80% of bacteria isolated from the rhizosphere (Loper and Schroth 1986); however, reports demonstrating production of IAA by gram-positive free-living soil bacteria are still lacking. Synthesis of IAA by the gram-positive phytopathogen *Rhodococcus fascians* recently was reported (Vandeputte et al. 2005). Gas chromatography-mass spectrometry (GC-MS) analysis verified gibberellin production by *B. pumilus* and *B. licheniformis* (Gutierrez-Mareño et al. 2001). We previously have shown that representatives of the *B. subtilis/amyloliquefaciens* group are able to produce substances with auxin (IAA)-like bioactivity. The presence of IAA-like compounds in the culture filtrates of several members of this group, including FZB42, was detected by enzyme-linked immunosorbent assay tests with IAA-specific antibodies, when those strains were grown at low temperature and low aeration (Idriss et al. 2004).

Using a combined approach of chemical and genetic analysis, we demonstrate here that biosynthesis of IAA in the PGPR *B. amyloliquefaciens* FZB42 affects its ability to promote plant growth. Moreover, this ability is dependent on the presence of tryptophan, which is one of the main compounds present in several plant exudates (Kamilova et al. 2006). Inactivation of genes involved in tryptophan biosynthesis and in a putative tryptophan-dependent IAA biosynthesis pathway lead to reduction of both IAA concentration and plant-growth-promoting activity in the respective mutant strains.

RESULTS

Plant-growth-promoting activity of FZB42 was demonstrated in a *Lemna minor*-based, microtiter-plate assay.

Diluted culture filtrates or growing cells of FZB42 were able to stimulate plant growth in a test system developed from the duck weed clone, *Lemna minor* ST, grown in 48-well microtiter plates (discussed below). However, this effect depends on the concentration of the added bacterial supernatant or cells. Di-

luted FZB42 culture filtrate at 0.1% caused a significant ($P \leq 0.01$) increase in fresh weight compared with the control plants. Addition of higher concentrations of bacterial culture filtrate inhibited plant growth (Table 1). The stimulating effect on plant growth was even higher when growing bacterial cells in appropriate concentration (2×10^5 CFU for maximal promotion) were added. Addition of higher numbers of bacteria ($>1 \times 10^7$ CFU) resulted in significant ($P \leq 0.01$) reduction of plant fresh weight. This assay was used throughout this study in order to analyze plant-growth-promoting activity of FZB42 and the mutant strains described in the later sections.

Identification and quantification of IAA by GC-MS.

Phytohormone-like action of culture filtrates obtained from *B. amyloliquefaciens* cells grown at 22°C and low aeration was demonstrated previously (Idris et al. 2004). In order to identify the chemical compound responsible for the plant-growth-promoting activity, culture filtrates of FZB42 were separated by analytic high-performance liquid chromatography (HPLC) and further analyzed by mass spectrometry, as described below. Full-scan GC-MS analyses of authentic IAA showed that the fragmentation pattern of this compound produced two main ions, m/z 202 and 261 (Gaskin and MacMillan 1991) at 9.02 min under the conditions used. In parallel analyses, culture filtrates of *B. amyloliquefaciens* exhibited similar chromatographic profiles with the same prominent ions. Furthermore, the spectra obtained in the scans at this retention time were identical to those produced by authentic IAA and those previously reported

for IAA (Gaskin and MacMillan 1991). Thus, although no evidence of the presence of gibberellins in the extracts was found, these analyses identified the presence of IAA in *B. amyloliquefaciens* and suggested that IAA might act as a main component of the plant-growth-promoting activity exerted by this strain.

For IAA quantitation in the initial extractions, IAA levels in the bacterial media were estimated from peak areas. Based on these results, different amounts of [$^2\text{H}_2$]-IAA were added as internal standard, and the extractions were repeated until the amount of internal standards and the amount of natural IAA were approximately equal. The experiment then was repeated under these conditions to obtain accurate quantifications. First, the effect on bacterial IAA production of adding tryptophan to the media was investigated. IAA content in culture filtrates was quantified by GC-MS of the ratio of ions at m/z 202 and 204 of IAA/[$^2\text{H}_2$]-IAA, respectively. Bacteria secreted relatively high IAA amounts; even when grown without Trp (29.3 ng ml $^{-1}$). In culture filtrates obtained from cells grown in the presence of tryptophan, IAA production was significantly higher. Bacterial media supplemented with 0.1 and 1 mM Trp yielded significantly different ($P < 0.05$) mean values of 51.0 and 54.0 ng ml $^{-1}$ of IAA, while a significant fivefold stimulation of IAA production, mean value 161.0, was registered when 5 mM Trp was added to the culture. These observations indicated that IAA production in *B. amyloliquefaciens* is tryptophan dependent.

This experiment also discarded hypothetical IAA contaminations of the bacterial media from plant extracts or laboratory manipulation, because IAA amounts were dependent upon Trp concentration.

Disruption of tryptophan biosynthesis genes results in lower IAA synthesis.

Production of IAA was dependent on tryptophan and strain FZB42 is genetically amenable (Koumoutsis et al. 2004); therefore, we performed knockout mutations to interrupt tryptophan biosynthesis in FZB42. Sequence analysis based on the whole genome sequence of *B. amyloliquefaciens* (Chen and Borriss; Koumoutsis et al. 2004; unpublished results) revealed complete co-linearity between the tryptophan biosynthesis gene clusters of *B. subtilis* (Henner and Yanofsky 1993) and FZB42 (AM295011). Function of the encoded gene products of the selected genes and their degree of similarity to *B. subtilis* are summarized in Table 2. Sequences containing parts of the *trpAB* (2.068 kb) and *trpED* (2.145 kb) genes were amplified with specific primers derived from the FZB42 genome se-

Table 1. Plant-growth-promoting activity of *Bacillus amyloliquefaciens* FZB42 on *Lemna minor*^w

Conditions	Concentration	Plant growth (mg/well) ^x
Without bacteria	...	14.1 ± 0.8
Culture filtrate ^y	0.1%	17.5 ± 1.0
	0.5%	12.0 ± 1.4
Growing cells ^z	2×10^5	19.3 ± 1.2
	1×10^7	13.1 ± 0.7

^w Diluted culture filtrates or growing cells were added to the *Lemna* plantlets. Plant growth is expressed as milligrams of plant fresh weight per well.

^x Mean ± standard error from at least three independent experiments.

^y Concentration is expressed as the final concentration of the *Bacillus* culture filtrate per well.

^z Concentration of growing cells represents the number of bacterial cells determined as CFU added to one micro titre plate well.

Table 2. *Bacillus amyloliquefaciens* FZB42 genes, possibly involved in tryptophan biosynthesis and indole-3-acetic acid (IAA) metabolism

Gene, FZB42 accession	Putative function	EC number	Amino acid number	pfam	Homologous gene	Organism	Identity	Similarity
Tryptophane ^y								
<i>trpE</i> , CAL26225.1	Anthranilate synthase	4.1.3.27	515	04715	BG10287	<i>Bacillus subtilis</i> 168	398/515 (77%)	445/515 (86%)
<i>trpD</i> , CAL26226.1	Anthranilate phosphoribosyltransferase	2.4.2.18	338	02885	BG10288	...	236/338 (69%)	259/338 (76%)
<i>trpA</i> , CAL26231.1	Tryptophan synthase α chain	4.2.1.20	267	00290	BG10291	...	204/263 (77%)	227/263 (86%)
<i>trpB</i> , CAL26229.1	Tryptophan synthase β chain	4.2.1.20	400	00291B	BG10290	...	337/400 (84%)	369/400 (92%)
Homologous ^z								
<i>ysnE</i> , CAL26203.1	IAA acetyltransferase	2.3.1.-	151	00583	P26945	<i>Azospirillum brasilense</i>	51/148 (34%)	75/148 (50%)
<i>dhaS</i> , CAL26192.1	NADP-dependent indole-3-acetaldehyde dehydrogenase	1.2.1.3	495	00171	AAC49575	<i>Ustilago maydis</i>	245/494 (49%)	335/494 (67%)
<i>yhxC</i> , CAL26199.1	Nitrilase, hydrolase carbon-nitrogen family	3.5.5.1	513	00795	AAB05220.1	<i>Arabidopsis thaliana</i>	31/105 (29%)	49/105 (46%)

^y Genes involved in tryptophane biosynthesis.

^z Genes homologous to genes involved in IAA biosynthesis.

quence. Disruption of the respective genes was achieved by insertion of an antibiotic resistance cassette via homologous recombination, leading to replacement of the wild-type gene via double crossover as previously described (Chen et al. 2006; Koumoutsis et al. 2004). Resulting transformants were confirmed by Southern hybridization and found to be unable to grow in minimal medium without tryptophan. When both *trp* mutant strains, E101 ($\Delta trpED$) and E102 ($\Delta trpAB$), were analyzed by GC-MS for the presence of IAA in their culture filtrates, a distinct reduction of IAA production was observed. Quantification revealed that both mutant strains produced less plant hormone than the wild-type strain. Strain E101 bearing the knockout mutation affecting the early steps of Trp biosynthesis (Table 3) produced only 15% of the wild-type strain IAA levels, indicating that the main route of IAA production in *B. amyloliquefaciens* is dependent on tryptophan biosynthesis.

IAA concentration in strains with knockout mutations in genes bearing homology to IAA biosynthesis genes.

Several routes of tryptophan-dependent IAA biosynthesis in microorganisms are known (Fig. 1); however, no gene products involved in IAA biosynthesis in gram-positive bacteria have been identified thus far. Examination of the whole-genome sequence of *B. amyloliquefaciens* FZB42 revealed three candidate genes with apparent homology to genes previously reported to be involved in IAA metabolism: *ysnE*, encoding a protein similar to the IAA acetyltransferase from *Azospirillum brasilense* (Zimmer et al. 1991); *dhaS*, similar to indole-3-acetaldehyde dehydrogenase from *Ustilago maydis* (Basse et al. 1996); and *yhcX*, encoding a putative nitrilase similar to nitrilase2 from *Arabidopsis thaliana* (Hillebrand et al. 1998). All three genes have counterparts in the genome of *B. subtilis* 168 (Kunst et al. 1997). Properties of the deduced gene products, identity, and similarity scores are summarized in Table 2.

The respective genes were inactivated by the gene replacement technique as described above. Amplification of *ysnE*, *dhaS*, and *yhcX* gene sequences was performed with sequence-specific primers designed according to the sequence information from AM295009 (*ysnE* containing region), AM295008 (*dhaS* containing region), and AM295010 (*yhcX* containing region) (Table 4). Integrative plasmids unable to replicate in *Bacillus* spp. and bearing the respective gene sequences interrupted by an antibiotic cassette were transformed after linear-

ization into competent *B. amyloliquefaciens* FZB42 cells. Transformants expressing the resistance marker after integration of the corresponding homologous flanking sequences within the chromosome via double crossover were analyzed by Southern hybridization.

Quantification of the IAA amounts present in culture filtrates of strains E103 ($\Delta ysnE$) and E105 ($\Delta yhcX$) by the GC-MS methodology revealed reduced amounts of IAA. The $\Delta ysnE$ mutant strain formed only 28% of the amount produced by the wild type, while the strain bearing the mutation $\Delta yhcX$ produced half of the amount of the wild type. Strain E104 ($\Delta dhaS$) was not significantly affected in its IAA production, suggesting no participation of the *dhaS* gene product in IAA synthesis (Table 5).

Survey of plant-growth-promoting activities in mutant strains revealed dependency on IAA production.

The Trp-auxotrophic mutant strains E101 ($\Delta trpED$) and E102 ($\Delta trpAB$) were analyzed for plant-growth-promoting activity in the *Lemna* test system as described above. Direct application of growing bacteria resulted in only a slight increase of plant fresh weight compared with the untreated control, whereas addition of diluted culture filtrates obtained from both *trp* mutant strains did not result in any promotion of plant growth. The strong reduction in or abolition of plant-growth-promoting activity in both *trp* mutants may be due to the severe reduction of IAA excretion in those strains (Table 5).

Strain E103 bearing the $\Delta ysnE$ mutation was impaired in its capability to support plant growth. This is in line with our hypothesis that IAA production is closely linked with plant growth promotion. Strain E104 ($\Delta dhaS$), producing nearly as much IAA as the wild type, retained its ability to promote plant growth. However, strain E105 ($\Delta yhcX$), forming approximately 50% IAA compared with the wild type, still was able to stimulate plant growth to the same extent as the wild type. The level of IAA produced by this strain is probably still above the critical threshold for supporting plant growth (Table 5). Alternatively, this observation is compatible with the notion that the promotion of plant growth may be due not only to the amount of bacterial IAA but also to the additional IAA converted by the *Lemna* nitrilase from the accumulated indole 3-acetonitrile excreted by the bacterium after blockage of IAA formation due to the knockout of the *yhcX* gene product.

Table 3. Bacterial strains and plasmids used

Strain or plasmid	Description ²	Source or reference
<i>Escherichia coli</i>		
DH5 α	<i>supE44, $\Delta lacU169$ ($\phi 80lacZ$ M15) <i>hsdR17 recA1 gyrA96 thi-1 relA91 endA1</i></i>	Lab strain
<i>Bacillus amyloliquefaciens</i>		
FZB42	Wild-type isolate	BGSC 10A6
E101	$\Delta trpAB::Em^r$	pEI2 \rightarrow FZB42
E102	$\Delta trpED::Em^r$	pEI4 \rightarrow FZB42
E103	$\Delta ysnE::Em^r$	pEI6 \rightarrow FZB42
E104	$\Delta dhaS::Em^r$	pEI8 \rightarrow FZB42
E105	$\Delta yhcX::Em^r$	pEI10 \rightarrow FZB42
Plasmids		
pGEM ^R -T	Cloning vector Amp ^r , <i>lacZ'</i>	Promega
pEI1	pGEMT containing 2.06 kb insert of <i>trpAB</i>	This work
pEI2	pEI1 with <i>trpAB::em^r</i> insertion	This work
pEI3	pGEMT containing 2.14 kb insert of <i>trpED</i>	This work
pEI4	pEI3 with <i>trpED::em^r</i> insertion	This work
pEI5	pGEMT containing 1.94-kb insert of <i>ysnE</i>	This work
pEI6	pEI5 with <i>ysnE::em^r</i> insertion	This work
pEI7	pGEMT containing 2.633-kb insert of <i>dhaS</i>	This work
pEI8	pEI7 with <i>dhaS::em^r</i> insertion	This work
pEI9	pGEMT containing 1.9-kb insert of <i>yhcX</i>	This work
pEI10	pEI9 with <i>yhcX::em^r</i> insertion	This work

² Em^r = erythromycin resistant and Amp^r = ampicillin resistant.

the *ipdc* gene product (indole-3-pyruvate decarboxylase), to demonstrate that IAA synthesis in bacteria is dependent on tryptophan concentration.

Because gene products involved in IAA metabolism in gram-positive bacteria are completely unknown, we applied a genomic approach, taking advantage of the whole genome sequence of *B. amyloliquefaciens* (Koumoutsi et al. 2004). Only three genes with apparent homology to genes already known to be involved in IAA biosynthesis in other organisms were detected. Mutants bearing *ΔysnE* and *ΔyhcX* deletions were found impaired in IAA production. The deduced *yhcX* gene product is similar to nitrilase2 of *Arabidopsis thaliana*, which catalyzes the direct conversion of indole 3-acetonitrile to IAA (Hillebrand et al. 1998). The *ysnE* gene is similar to a putative IAA acetyl transferase gene localized within the tryptophan biosynthesis gene cluster of the plant-growth-promoting α proteobacterium *Azospirillum brasilense* (Zimmer et al. 1991). Its deduced gene product belongs to a widely distributed family of acetyl transferases (acetyltransf_1) that catalyze the transfer of an acetyl group to a nitrogen atom on the acceptor molecule. YsnE has been suggested to participate in the tryptophan-dependent IAA production (Zimmer et al. 1991). Taken together, the results present additional evidence for the existence of a tryptophan-dependent pathway as the main route of IAA biosynthesis in *B. amyloliquefaciens* FZB42. However, minor tryptophan-independent pathways for IAA biosynthesis may exist in FZB42, because IAA biosynthesis was not abolished completely in the *trp*, *ysnE*, and *yhcX* mutant strains. Double mutants might be useful to prove the presence of alternative Trp-independent pathways.

As with plants, decarboxylation and deamination of tryptophan seem to be the preferred routes in bacterial IAA metabolism (Normanly and Bartel 1999). Phytopathogenic and plant-beneficial gram-negative bacteria synthesize IAA by Trp-dependent pathways with indole-3-pyruvic acid (IPA), indole-3-acetamide (IAM), or indole-3-acetonitrile (IAN) as important intermediates (Kobayashi et al. 1995; Koga et al. 1991; Magie et al. 1963; Patten and Glick 1996) (Fig. 1). IAA synthesis in gram-positive bacteria has been detected in *Paenibacillus polymyxa* (Lebuhn et al. 1997) and investigated in more detail in the phytopathogenic *Rhodococcus fascians*. The main biosynthetic route for IAA in this bacterium is the IPA pathway, with a possible rate-limiting role for indole-3-ethanol (Vandeputte et al. 2005). The results presented here do not support such a scenario for IAA metabolism in *B. amyloliquefaciens*. The knockout mutant with a destroyed function of the *dhaS* gene was not impaired in IAA biosynthesis in FZB42. DhaS is similar to aldehyde dehydrogenase, probably catalyzing the last reaction of the indole-pyruvic acid pathway converting indole-3-acetaldehyde to IAA in *U. maydis* (Basse et al. 1996). No gene products similar to the key enzymes of the IPA pathway, indole pyruvate decarboxylase (IPDC) and tryptophan transaminase (converting tryptophan to indole-3-pyruvic acid) (Patten and Glick 1996), were detected during our analysis of the whole-genome sequence of FZB42. Genes with similarity to Trp monooxygenase (TMO) and to indole-3-acetamide hydrolase (IAMH) catalyzing both reactions of the IAM pathway could not be detected. Therefore, feeding experiments will be necessary to uncover the metabolic route for tryptophan-dependent IAA synthesis in FZB42, and the roles of the reactions catalyzed by the *ysnE* and *yhcX* gene products in IAA biosynthesis remain to be elucidated.

When mutant strains (*AtrpDE*, *AtrpAB*, and *ΔysnE*) with low IAA synthesis capacity were added to duckweed fronds, plant-growth-promoting effects decreased, demonstrating a close correlation of plant growth promotion and auxin production in FZB42. It is likely that tryptophan-like compounds

present in plant exudates stimulate IAA synthesis of the PGPR colonizing plant surface structures. In fact, our successful attempts to reisolate *B. amyloliquefaciens* directly from *L. minor* plantlets (E. E. Idris and R. Borriss, unpublished) indicates that the bacteria living in close vicinity to the plant surface can uptake excreted plant compounds. Recently, it has been shown that one of the main compounds present in some plant exudates is L-tryptophan (Kamilova et al. 2006). One could speculate that stimulated bacterial IAA production in the presence of the IAA precursor tryptophan will lead vice versa to further promotion of plant growth, suggesting a close symbiotic relationship between the plant and colonizing FZB42. Simultaneously, these findings provide a possible explanation for the remarkably high rhizosphere competence observed in FZB42 growing on tomato seedlings (Cadena-Cepeda et al. 2006). The ability to colonize plant roots may depend to some degree on the capability of the bacterium to synthesize IAA. It has been proposed that bacterial IAA synthesis contributes to enhanced rhizosphere competence by i) detoxification of Trp analogues present on host plant surfaces (Lebuhn et al. 1997) and ii) stimulation of the release of plant exudates (Lambrecht et al. 2000), the downregulation of plant defense (Yamada 1993), or the inhibition of the hypersensitive response of infected plants (Robinette and Matthyse 1990). However, further experiments are necessary to confirm such a scenario in the case of *B. amyloliquefaciens*.

In conclusion, the results presented here elucidate the role of Trp-dependent IAA synthesis within the spectrum of the diverse, mainly unknown molecular effects exerted by root-colonizing bacilli on plant growth.

MATERIALS AND METHODS

Strains and growth conditions.

Bacterial strains used in this study are listed in Table 3. *B. amyloliquefaciens* FZB42 was deposited as strain 10A6 in the culture collection of the Bacillus Genetic Stock Center. Bacteria were cultivated routinely in Luria broth (LB) solidified with 1.5% agar. For IAA production, the bacteria were grown for 72 h in Landy medium (Landy et al. 1948) at 25°C and 75 rpm (Idris et al. 2004). Mutant strains deficient in Trp biosynthesis routinely were grown in the presence of Trp at 20 μ g/ml. The duckweed clone *L. minor* ST originally was isolated by Pirson and Seidel (1950) and was delivered from the culture collection of the Botanical Institute of the University of Jena, Germany.

Table 5. Effect of knockout mutations on indole-3-acetic acid (IAA) production and plant growth promoting activity^z

Genotype	Percentage of wild type	
	IAA production	Growth promotion
E101 <i>AtrpED</i> ::Em ^r	14.7 d	16.8 b
E102 <i>AtrpBA</i> ::Em ^r	38.0 c	19.2 b
E103 <i>ΔysnE</i> ::Em ^r	28.9 c	19.1 b
E104 <i>ΔdhaS</i> ::Em ^r	71.2 a	71.0 a
E105 <i>ΔyhcX</i> ::Em ^r	51.4 b	81.5 a

^z IAA concentration was determined after growth in Landy medium without Trp except the two *trp* mutants cultivated in the presence of Trp at the rate of 20 μ g/ml. Abundance of IAA was calculated through gas chromatography-mass spectrometry quantitation of the area ratio of ions at m/z 202 and 204 of IAA/[²H₂]-IAA, respectively. Plant growth promotion was assayed in the *Lemna minor* test system, using 2 \times 10⁵ cells per well. Diluted culture filtrates (final concentration of 0.1%) from *trp*-minus mutants did not significantly affect growth of *Lemna* plants. Em^r = erythromycin resistant. Data are means and different letters in the same column indicate significant differences (*P* < 0.05).

Preparation of competent *B. amyloliquefaciens* FZB42 cells and DNA transformation.

Competent cells of *B. amyloliquefaciens* were obtained by modifying the two-step protocol of Kunst and Rapoport (1995). Cells were grown overnight in LB medium at 28°C (170 rpm) and were diluted the next day in GCHE medium containing 1% glucose, 0.2% potassium L-glutamate, 100 mM potassium phosphate buffer (pH 7), 3 mM trisodium citrate, 3 mM MgSO₄, 22 mg of ferric ammonium citrate per liter, 50 mg L-typtophan per liter, and 0.1% casein hydrolysate, to an optical density at 600 nm (OD₆₀₀) of 0.3. The cell culture then was incubated at 37°C under vigorous shaking (200 rpm) to midexponential growth (OD₆₀₀ of approximately 1.4). Dilution with an equal volume of GC medium (GCHE medium without potassium glutamate and casein hydrolysate) followed and the cells were incubated further under the same conditions for 1 h. The culture then was divided into two tubes and cells were harvested by centrifugation at 6,000 rpm for 5 min (room temperature). Afterwards, the pellets were resuspended in 200 µl of the supernatant and 1 µg of DNA in 2 ml of transformation buffer consisting of 15 mM (NH₄)₂SO₄, 80 mM K₂HPO₄, 45 mM H₂KPO₄, 35 mM sodium citrate, 1 mM EGTA, 25 mM glucose, and 30 mM MgCl₂. After incubation at 37°C under shaking at 75 rpm for 20 min, 1 ml of LB medium containing a sublethal concentration (0.1 µg/ml) of the appropriate antibiotic was added. The cells were grown under vigorous shaking for 90 min and plated on selective agar plates. For selection of erythromycin-resistant transformants, erythromycin at 1 µg/ml and lincomycin at 25 µg/ml were added to the selection plates.

The *Lemna* biotest system.

L. minor ST was propagated axenically in filter-sterilized (0.45 to 0.2 µm) Steinberg medium adjusted to pH 5.5. The growth medium consisted of the following components: 3.46 mM KNO₃, 1.25 mM Ca(NO₃)₂ · H₂O, 0.66 mM KH₂PO₄, 0.072 mM K₂HPO₄, 0.41 mM MgSO₄, 1.94 µM H₃BO₃, 0.63 µM ZnSO₄ · 7 H₂O, 0.18 µM Na₂MoO₄ · 2 H₂O, 0.91 µM MnCl₂ · 4 H₂O, 2.81 µM FeCl₃ · 6 H₂O, and 4.03 µM EDTA. Under aseptic conditions, four plants with two to three budding-pouches (fronds) were incubated in 200 ml of medium in a 500 ml-conical flask. Flasks were kept at 20°C with continuous light until a sufficient number of homogenous *Lemna* plants were obtained. The growth medium was changed each week. To prove the bioeffects of FZB42 and its derivatives on *Lemna* spp. growth, a model test system based on duckweed growing in 48-well microtiter plates was developed. Each well contained 1.2 ml of Steinberg minimal medium. Standardized *Lemna* plants possessing two fronds were transferred aseptically into every well of the microtiter plate. Culture filtrates or bacteria in appropriate dilutions were added directly. Each variant was performed in six replicates. The microtiter plates were kept at 20°C and 24 h of light for 10 days. Plants were harvested and growth was determined by determination of fresh weight and frond number. The result of each trial was confirmed by four repetitions.

Construction of plasmids and strains.

In order to replace the respective wild-type genes, appropriate gene cassettes consisting of the erythromycin resistance determinant flanked by the respective gene sequences were constructed (Table 1). Primers for amplification of chromosomal DNA sequences were designed according to sequence information obtained from the sample genome sequence of *B. amyloliquefaciens* as previously reported (Koumoutsi et al. 2004) (Table 4). Briefly, the amplified sequences were cloned into the pGEMT vector. A unique restriction site located within the central part of the coding region was used to interrupt the gene se-

quence by insertion of the *ermAM* resistance determinant isolated from plasmid pDB101 (Ceglowski and Alonso 1994).

Identification and quantification of IAA by GC-MS.

Extraction and purification. Culture filtrates of FZB42 and its Trp-minus mutants, produced in Landy medium under specific conditions, at 22°C and 75 rpm in the dark (Idris et al. 2004), were used for plant hormone identification. Extractions were performed with 80% methanol. After purification with SS-Sax and C₁₈ Sep-Pak cartridges (Talon et al. 1990), samples were dried to vacuum until further fractionation with reverse-phase HPLC. Abscisic acid (1 µg) then was added as an internal standard to ascertain the reproducibility of retention times. Samples were filtered through a 0.45-µm-diameter nylon filter and were injected into a Waters high-performance liquid chromatograph. The instrument was equipped with an analytical column (25 by 0.46 cm i.d.) packed with Hypersil C18 and attached to a C18 Guard-Pak precolumn. A 40-min linear gradient of 20 to 100% methanol in 1% aqueous acetic acid at a flow rate of 1 ml min⁻¹ was used. The HPLC fractions were collected at 1-min intervals and conveniently grouped. Samples were evaporated to dryness, methylated with ethereal diazomethane, and trimethylsilylated at room temperature for at least 1 h with 5 to 10 µl of bis-trimethylsilyl-trifluoroacetamide.

Identification. All HPLC fractions were analyzed by GC-MS using a gas chromatograph (Star 3400 CX; Varian, Sunnyvale, CA, U.S.A.) coupled to an ion trap mass spectrometer (Saturn 3; Varian). The samples (1 µl) were co-injected automatically with an autosampler (8200 CX; Varian) in the splitless mode into a fused silica capillary column (30 m by 0.25 mm by 0.25 µm of film thickness) (DB5MS; J&W Scientific, Folsom, CA, U.S.A.). The oven temperature was 50°C and, after 1 min, was increased at 30°C min⁻¹ to 240°C and then at 10°C min⁻¹ to 280°C. The He inlet pressure was 60 kPa and the injector, interface and manifold temperatures were 280, 290, and 170°C, respectively. Other operating parameters were as described by Ben-Cheikh and associates (1997). For hormone identification, full-scan positive ion-electron-impact mass spectra were acquired scanning from 100 to 600 amu at 1 s per scan cycle, a range including spectra of most acidic plant growth regulators, such as indole-acetic acid and gibberellins. IAA production was verified by comparing HPLC elution volumes, KRIs (Kovats retention indices), and mass spectra with those of authentic IAA and published data (Gaskin and MacMillan 1991).

Quantitation. For quantitation purposes, [²H₂]-IAA was added to the extracts as an internal standard and the above procedures for extraction, purification, and GC-MS analyses were repeated. Abundance of IAA was monitored through calculation of the area ratio of ions at m/z 202 and 204 of IAA/[²H₂]-IAA, respectively, in the chromatographic profiles. In preliminary analyses, various amounts of internal standards were added to the extracts in consecutive extractions and, thus, the produced IAA amounts initially were estimated. This strategy was repeated until the amount of internal standards and the amount of endogenous IAA was approximately equal, which is a requirement of the calculation method (Talon and Zeevaert 1992). Once the amount of internal standard to be added was established, final determinations on independent extractions were carried out (Mehouachi et al. 2000). In these final quantitations, several injections that provided near-identical results for each sample were performed. All quantitations were repeated independently on at least two different culture replicates and only averages of these final quantitations are presented.

Nucleotide sequence accession numbers.

The GenBank accession numbers for the *B. amyloliquefaciens* FZB42 typtophan biosynthetic gene cluster and the DNA

fragments containing the *dhaS* gene, the *ysnE* gene, and the *yhxC* gene are AM295011, AM295008, AM295009, and AM295010, respectively.

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