JOURNAL OF BACTERIOLOGY, Mar. 1989, p. 1718–1724
0021-9193/89/031718-07$02.00/0
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Vol. 171, No. 3

Molecular Cloning of a Gene for Indole-3-Acetamide Hydrolase from *Bradyrhizobium japonicum*

MASAMI SEKINE,[1]* KAZUTADA WATANABE,[2] AND KUNIHIKO SYŌNO[1]

Department of Pure and Applied Sciences, College of Arts and Sciences, The University of Tokyo, Meguro-ku, Tokyo 153,[1] and Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo 173,[2] Japan

Received 21 March 1988/Accepted 15 December 1988

A pLAFR1 cosmid genomic library of wild-type *Bradyrhizobium japonicum* J1063 was constructed. A cosmid clone designated pBjJ4, containing a 26-kilobase (kb) DNA insert, was identified as being able to confer the ability to convert α-naphthaleneacetamide to α-naphthaleneacetic acid on *B. japonicum* J1B7 Rif^r, which cannot perform this conversion. The gene coding for the enzyme that converts α-naphthaleneacetamide to α-naphthaleneacetic acid was localized in the 3.5-kb region of pBjJ4 by recloning in plasmid pSUP202. The gene coding for the enzyme was also mapped by Tn*5* insertion mutagenesis to a region of ca. 2.3 kb. When the gene was placed behind the *lacZ* promoter and used to transform *Escherichia coli*, a high level of expression of indole-3-acetamide hydrolase activity was found. Since there have been no reports of this activity in *E. coli*, we have thus confirmed that the gene cloned here is a structural gene for indole-3-acetamide hydrolase and have designated it as the *bam* (*Bradyrhizobium* amidehydrolase) gene. Southern hybridization with the central region of the *bam* gene indicated that a high degree of similarity exists among the *bam* gene, the *iaaH* gene from *Pseudomonas savastonoi*, and the *tms-2* gene from *Agrobacterium tumefaciens*. The result suggests that there is a common origin for the gene that encodes the enzyme that catalyzes the biosynthesis of indoleacetic acid.

It has been shown that bacterial production of the phytohormone indoleacetic acid (IAA) is involved in the virulence of several interactions between microorganisms and plants (19). Several pathways have been reported for the conversion of tryptophan to IAA in bacteria (27). The best-studied pathways involve conversion via indole-3-acetamide (IAM) (the IAM pathway) in *Pseudomonas savastanoi* and in *Agrobacterium tumefaciens*. In these pathways, tryptophan 2-monooxygenase converts tryptophan to IAM, and then indole-3-acetamide hydrolase catalyzes the conversion of IAM to IAA.

P. savastanoi is a pathogen that infects olive and oleander plants, inducing tumorous outgrowths called olive (or oleander) knots. The formation of galls is dependent on the bacterial production of IAA, and the severity of the disease is correlated with the amounts of IAA secreted by bacteria through the IAM pathway (4, 5). The genes for the IAM pathway, termed the *iaaM* and *iaaH* genes, are organized in an operon which is located on a pIAA plasmid and are expressed only in bacteria (4, 5).

A. tumefaciens induces tumors called crown gall on most dicotyledonous plants by integration of DNA into the plant genome and subsequent expression of a portion of the Ti plasmid, the transferred DNA (T-DNA) (20). Non-auxin-dependent growth of tumors is responsible for high concentrations of IAA produced by the IAM pathway located in the T-DNA (19). The T-DNA genes *tms-1* and *tms-2* encode enzymes for the IAM pathway and are functional only in plants (11). Since the IAM pathway is not found in plants (21), the origin of this pathway is of great interest with respect to the evolutionary relationship between procaryotic and eucaryotic genes.

Members of the genera *Rhizobium* and *Bradyrhizobium* belong to the family *Rhizobiaceae*, which includes the genera *Agrobacterium*, and induce nitrogen-fixing root nodules on their host legumes. It is generally accepted that IAA is produced in free-living cultures of *Rhizobium* spp. and *Bradyrhizobium* spp. (1, 2, 8, 14, 32). We have previously suggested that the IAM pathway is present in *Bradyrhizobium* spp. (22). Our interest is focused on whether the IAM pathway in *Bradyrhizobium* spp. is involved in root nodule formation. The identification and isolation of the genes involved in the IAM pathway will allow a detailed analysis of the physiological function of the pathway. The existence of the IAA biosynthetic pathway, via indole-3-pyruvic acid, in *Bradyrhizobium japonicum* has been already reported (14). Thus, it is necessary to distinguish the IAM pathway from the indole-3-pyruvic acid pathway in order to clone the genes involved in the IAM pathway. The detection of the enzymatic activity that converts α-naphthaleneacetamide (NAM) to α-naphthaleneacetic acid (NAA), which is identical to indole-3-acetamide hydrolase activity (22), permits discrimination between the two different pathways. Therefore, we attempted to isolate the gene that encodes the enzyme that converts NAM to NAA in order to clone the gene for indole-3-acetamide hydrolase from *B. japonicum*.

In the present study, a 3.5-kilobase (kb) DNA fragment was isolated from *B. japonicum* J1063, which contains a gene that encodes the enzyme that catalyzes the conversion of NAM to NAA, and we characterized this gene further by Tn*5* mutagenesis. Additionally, we have detected indole-3-acetamide hydrolase activity in *Escherichia coli* transformed with recombinant plasmids that carry the cloned DNA fragment and have further characterized this activity by deletion mutagenesis. In addition, Southern hybridization with the central region of the *bam* gene against the *iaaH* and *tms-2* genes indicated that a high degree of similarity exists among these three genes.

* Corresponding author.

TABLE 1. Bacteria and plasmids

Bacterial strain or plasmid	Relevant characteristics[a]	Source or reference(s)[b]
B. japonicum		
J1063	Wild-type strain	22
J1B7 Rif	Rif^r derivative of wild-type strain J1B7	This study
E. coli		
HB101	*recA hsdR hsdM pro* Str^r	3
MM294	*hsdR thi endA*	6
S17-1	MM294[RP4-2 (Tc^r::Mu) (Km^r::Tn*7*) Tra (IncP) Tp^r Sm^r	23
DB1572	*recA lacZ*::Tn*5*	D. E. Berg
JM109	*recA hsdR thi endA*	34
Plasmids		
pLAFR1	IncP Tc^r	12
pRK290	IncP Tc^r	6
pRK2013	ColE1::pRK2 Tra$^+$ Km^r	6
pSUP202	Mob$^+$ Ap^r Cm$^+$ Tc^r	24, 25
RP4-4	Tra$^+$ Mob$^+$ Ap^r Tc^r	24, 25
Bluescript KS M13$^+$	Ap^r	SCS
Bluescript SK M13$^+$	Ap^r	SCS
pBjJ4	pLAFR1 carrying the *bam* gene of *B. japonicum* J1063; Tc^r	This study
pRBj7	pRK290 carrying the *bam* gene of *B. japonicum* J1063; Tc^r	This study
pIM8	pSUP202 carrying the 8-kb *Hin*dIII fragment of pBjJ4; Ap^r Cm^r	This study
pIM14	pSUP202 carrying the 13-kb *Hin*dIII fragment of pBjJ4; Ap^r Cm^r	This study
pIM6	pSUP202 carrying the 6.5-kb *Cla*I fragment of pIM14; Ap^r Cm^r	This study
pIM61	pSUP202 carrying the 3.5-kb *Xho*I-*Cla*I fragment of pIM6; Ap^r Cm^r	This study
pIM62	pSUP202 carrying the 4.3-kb *Sal*I-*Cla*I fragment of pIM6; Ap^r Cm^r	This study
pKS16	Bluescript KS M13$^+$ carrying the 3.5-kb *Xho*I-*Cla*I fragment of pIM6; Ap^r	This study
pSK16	Bluescript SK M13$^+$ carrying the 3.5-kb *Xho*I-*Cla*I fragment of pIM6; Ap^r	This study
pSK161	Derivative of pSK16; Ap^r	This study
pSK162	Derivative of pSK16; Ap^r	This study
pSK163	Derivative of pSK16; Ap^r	This study
pSK164	Derivative of pSK16; Ap^r	This study
pSK165	Derivative of pSK16; Ap^r	This study
pCP3	pBR328 carrying the 4.0-kb *Eco*RI-*Sal*I fragment bearing the *iaaM* and *iaaH* genes; Ap^r	33
pSP64tms2	pSP64 carrying the 2.0-kb *Hin*dIII fragment bearing the *tms-2* gene; Ap^r	This study

[a] Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Rif, rifampin; Sm, streptomycin; Tc, tetracycline; Tp, trimethoprim.
[b] SCS, Stratagene Cloning Systems.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. *B. japonicum* strains were grown at 28°C in TY medium, which consisted of (per liter) 5 g of tryptone (Difco Laboratories, Detroit, Mich.) and 3 g of yeast extract. *E. coli* strains were grown in LB medium (17) at 37°C. *E. coli* JM109 transformed with ligated DNA was grown on plates of M9 minimal glucose medium (17) supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml), isopropyl-β-D-thiogalactoside(IPTG) (0.2 mM), and ampicillin (50 μg/ml) to permit selection of colonies of *E. coli* cells that contained the recombinant plasmids. The antibiotics used in media for growth of *E. coli* were as follows (in micrograms per milliliter): ampicillin, 50; chloramphenicol, 30; tetracycline, 10; and kanamycin, 50. The antibiotics used in media for the growth of *Bradyrhizobium* strains were as follows (in micrograms per milliliter): carbenicillin, 100; chloramphenicol, 25; tetracycline, 10; kanamycin, 50; and rifampin, 100.

Isolation of DNA. Plasmid DNA from *E. coli* was isolated by the procedure of Holmes and Quigley (13). Total DNA was isolated from *B. japonicum* by the method described by Masterson et al. (18). In some cases, plasmid DNA was further purified by centrifugation through cesium chloride-ethidium bromide density gradients.

Restriction endonucleases and gel electrophoresis. Restriction endonucleases, calf intestinal alkaline phosphatase, and T4 DNA ligase were purchased from Takara Shuzo Co., Kyoto, Japan; Bethesda Research Laboratories, Rockville, Md.; and Boehringer GmbH, Mannheim, Federal Republic of Germany. All enzymatic reactions were performed essentially as recommended in the specifications of the manufacturers. Restriction fragments of DNA were analyzed by horizontal agarose gel electrophoresis in Tris-acetate buffer (40 mM Tris hydrochloride, 20 mM sodium acetate, 1 mM EDTA [pH 7.8]).

Transfer of DNA to filters and hybridization conditions. DNA fragments were transferred from agarose gels to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) by the Southern blot procedure (26). Hybridization was performed at 68°C as described by Maniatis et al. (17). After hybridization, the nitrocellulose filters were washed three times at 68°C in 0.2× SSC (1× SSC is 15 mM sodium citrate and 150 mM NaCl [pH 7.0]) containing 0.5% sodium dodecyl sulfate for high-stringency washing. Hybridized filters were exposed to Fuji HR-S100 film (Fuji Film, Tokyo, Japan) for 1 day with a Dupont Cronex Lightning-Plus intensifying screen.

Labeling of DNA. DNA preparations were labeled by the procedure of Feinberg and Vogelstein (9, 10) by using a kit

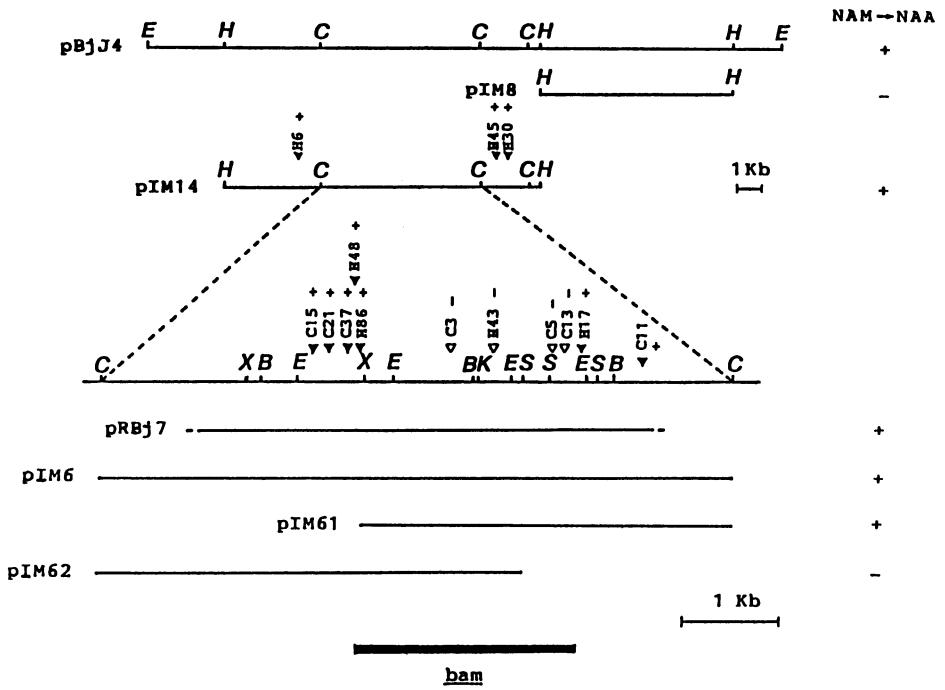

FIG. 1. Restriction endonuclease map of *B. japonicum* DNA of pBjJ4 and locations of the Tn*5* insertions. The arrowheads indicate the approximate locations of Tn*5* insertions in pIM14 (indicated as H6, H17, H30, H43, H45, and H86) and pIM6 (indicated as C3, C5, C11, C15, C21, and C37). Also indicated is whether Tn*5* insertions in plasmids could (+) or could not (−) confer the ability to enzymatically convert NAM to NAA on *B. japonicum* J1B7 Rif. Only relevant restriction sites are shown. These sites are represented as follows: B, *Bam*HI; C, *Cla*I; E. *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I; and X, *Xho*I.

from Amersham with [α-^{32}P]dCTP (New England Nuclear Corp., Boston, Mass.).

Construction of a *B. japonicum* cosmid library. A partial *Eco*RI digest of total DNA from *B. japonicum* J1063 was size-fractionated on a 10 to 40% sucrose density gradient by centrifugation at 24,000 rpm for 20 h in an SW27 rotor (Hitachi). Fractions (0.5 ml each) were collected, and those fractions that contained DNA fragments between approximately 22 and 35 kilobases (kb) in size were pooled and dialyzed against TE buffer (10 mM Tris hydrochloride, 1 mM EDTA [pH 7.5]) overnight. The DNA was then ligated to the *Eco*RI site of the cosmid vector pLAFR1 (12) at a ratio of 8 to 1 by weight. The ligated DNA was packaged into bacteriophage lambda by using a DNA packaging kit (Gigapack; Stratagene Cloning Systems, San Diego, Calif.) according to the manufacturer's instructions. The lambda phage containing the recombinant cosmids were used to infect *E. coli* HB101 grown in LB medium with 0.4% maltose. The cells were then plated on LB medium that contained tetracycline to select for transductants. Approximately 1,500 tetracycline-resistant clones were obtained, and more than 95% of the clones contained inserts; the average size of inserts was about 27 kb.

Subcloning and construction of plasmids. The DNA used for restriction mapping was prepared from the *dam*$^+$ strain of *E. coli* JM109. Plasmid pRBj7 was constructed by partial digestion with *Sau*3A of cosmid pBjJ4 (Fig. 1). The DNA fragments in the size range of ca. 4 to 8 kb were collected on DEAE-cellulose paper (31) after electrophoresis through 0.7% agarose gels, and the resulting DNA was ligated to the broad-host-range plasmid pRK290, which had been digested with *Bgl*II and treated with calf intestinal alkaline phosphatase (6). Transformants of *E. coli* HB101 treated with the ligated DNA were selected on LB plates that contained tetracycline.

Plasmids pIM14 and pIM8 were constructed in the following way. Cosmid pBjJ4 was digested with *Hin*dIII and subjected to electrophoresis through 0.7% low-melting-point agarose gels (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The appropriate bands (*Hin*dIII fragment of 13.0 and 8 kb) were cut out and extracted from the gels. The DNA samples were then ligated separately into pSUP202 (24, 25), which had been digested with *Hin*dIII and used to transform *E. coli* S17-1. Transformants were selected on LB plates that contained ampicillin.

Plasmid pIM6 was constructed by cloning the 6.5-kb *Cla*I fragment of pIM14 into pSUP202, which had been digested with *Cla*I. Plasmids pIM61 and pIM62 were derived from pIM6. The 3.5-kb *Xho*I-*Cla*I fragment and the 4.3-kb *Sal*I-*Cla*I fragment of pIM6 were cloned into pSUP202, which had been digested with *Xho*I and *Cla*I, and into pSUP202, which had been digested with *Sal*I and *Cla*I, respectively.

Plasmids pSK16 and pKS16 were constructed by ligating the 3.5-kb *Xho*I-*Cla*I fragment of pIM61 into the *Xho*I-*Cla*I sites of Bluescript SK M13$^+$ and Bluescript KS M13$^+$, respectively (Stratagene Cloning Systems). Plasmids were then tested by transformation of *E. coli* JM109. Transformants were selected on X-Gal (Boehringer GmbH) indicator plates, on which the desired transformants formed white colonies.

The plasmid pSK16, digested with *Hin*dIII and *Pst*I, was the substrate for the construction of the deletion derivatives pSK161, pSK162, pSK163, pSK164, and pSK165, which were prepared by the unidirectional deletion procedure with exonuclease III and mung bean nuclease by using a kit from Takara Shuzō, according to the specifications of the manufacturer.

Tn*5* mutagenesis. Transposon Tn*5* mutagenesis of inserts of DNA from *B. japonicum* J1063 in plasmids pIM6 and pIM14 was carried out in *E. coli* DB1572 by the procedure

for multicopy plasmids described by Simon (23). The sites of insertion of Tn*5* in the plasmids were determined from analysis of restriction fragments after digestion by *Bam*HI, *Hin*dIII, or *Xho*I.

Bacterial conjugation and selection of exoconjugants. The cosmid clones and the derivatives of pRK290 were mobilized from *E. coli* HB101 into *B. japonicum* J1B7 Rif by triparental matings by using pRK2013 as the helper plasmid (6). Exconjugants were selected on TY plates that contained rifampin and tetracycline. The derivatives of pSUP202 and the Tn*5*-mutated plasmids were mobilized into *B. japonicum* J1B7 Rif^r by biparental matings using mobilizing *E. coli* S17-1 as donor (23, 24). Exconjugants were selected on TY plates that contained either rifampin plus kanamycin (in the case of the Tn*5*-mutated plasmids) or rifampin plus chloramphenicol (for counterselection against the *E. coli* donor). Single colonies were then picked and used to analyze the ability of the various mutants to convert NAM to NAA.

Purification procedure and analysis for NAA and IAA. The assay for the activity that converts NAM to NAA was described in a previous paper (22). In experiments to analyze the enzymatic conversion of IAM to IAA, *E. coli* JM109, containing one of the recombinant plasmids at a time, was grown overnight at 37°C in 10 ml of liquid LB medium supplemented with 5 μl of IAM (10 mg/ml) and 25 μl of IPTG (100 mM). Extraction with dichloromethane and high-performance liquid chromatographic analysis for IAA were performed as described previously (22).

RESULTS

Screening of cosmid clones capable of the enzymatic conversion of NAM to NAA. In our previous paper (22), we demonstrated that indole-3-acetamide hydrolase activity can be detected by monitoring the conversion of NAM to NAA, and we developed a simple method for identifying the NAA generated from NAM by high-performance liquid chromatographic analysis. We examined the enzymatic activity in several strains of *B. japonicum* and found that *B. japonicum* J1B7 exhibited no conversion activity (22). To clone a gene that encodes the enzyme which converts NAM to NAA, we constructed a cosmid library of the genome of *B. japonicum* J1063, which possesses the enzyme that converts NAM to NAA. The cosmid clones were then mobilized from *E. coli* HB101 into the recipient *B. japonicum* strain, J1B7 Rif, which was then screened for positive clones, those with the ability to enzymatically convert NAM to NAA. *B. japonicum* J1B7 Rif is a rifampin-resistant mutant derived from *B. japonicum* J1B7. Approximately 500 clones from the *B. japonicum* cosmid bank were screened for the ability to convert NAM to NAA. About 100 cosmid clones were mixed as a group and mobilized into the recipient to examine the conversion activity. One of these groups conferred the conversion activity on *B. japonicum* J1B7 Rif (data not shown). This group was divided into 10 subgroups, each of which consisted of 10 clones. When the clones of each subgroup were mixed together and mobilized into the recipient, one subgroup was found to be able to confer the conversion activity on the recipient. Each clone of the conversion-positive group was mobilized separately into the recipient, and finally one clone, which was designated pBjJ4 and which contained a 26-kb insert, was identified and isolated (Fig. 1). The results indicate that cosmid clone pBjJ4 contains a gene that encodes the enzyme, presumably indole-3-acetamide hydrolase, that catalyzes the conversion of NAM to NAA or a locus that regulates the conversion activity.

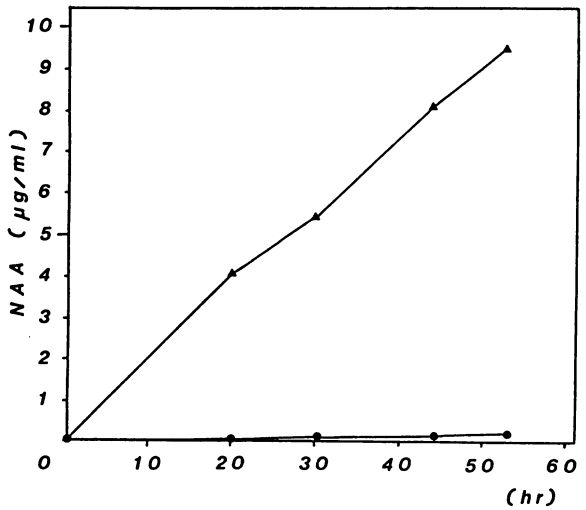

FIG. 2. Time course of NAA accumulation by the enzymatic activity that catalyzes the conversion of NAM to NAA in culture over a 53-h growth period. The medium was inoculated at time zero with bacterial cells and monitored at designated time points for cell density and NAA accumulation. The value at each time point is an average of two trials. Procedures for measuring NAA were those described in a previous paper (22). Symbols: ●, *B. japonicum* J1B7 Rif^r; ▲, *B. japonicum* J1B7 Rif harboring pBjJ4.

We next examined the time course of accumulation of NAA when *B. japonicum* J1B7 Rif was transformed with pBjJ4 (Fig. 2). While both strains showed similar growth rates, *B. japonicum* J1B7 Rif harboring pBjJ4 accumulated large amounts of NAA but plasmidless *B. japonicum* J1B7 Rif did not accumulate NAA during the 53-h growth period (Fig. 2). Thus, *B. japonicum* J1B7 Rif harboring pBjJ4 can constantly convert NAM to NAA.

If the NAM-to-NAA conversion activity could be expressed in *E. coli*, all subsequent steps could be performed by using *E. coli* cells. Therefore, we examined in liquid cultures the conversion activity of *E. coli* HB101 cells transformed with pBjJ4. There was no detectable activity in these cultures under our assay conditions.

Subcloning and restriction mapping. To subclone the gene which confers the conversion activity on *B. japonicum* J1B7 Rif from pBjJ4, the following two strategies were adapted.

The first strategy involved the preparation of a *Sau*3A partial digest of pBjJ4. DNA fragments in the size range of about 4 to 8 kb were subcloned into the *Bgl*II site of pRK290 in *E. coli* HB101. Ten transformants were mixed as a group and mobilized into *B. japonicum* J1B7 Rif. Mobilization of 120 clones resulted in identification of 2 clones which conferred the conversion activity on the recipient. One of the clones (pRBj7) contained a shorter insert (ca. 4.7 kb), and thus the clone was characterized further by restriction mapping. The insert fragment of pRBj7 could not be cut by *Bgl*II at either end.

A second strategy was subsequently used. When pBjJ4 was digested with *Hin*dIII, two fragments (13.0 and 8.0 kb) were produced, and these two *Hin*dIII fragments were cloned into pSUP202 to generate plasmids pIM14 and pIM8, respectively. pSUP202 is a mobilizable *E. coli* vector plasmid that contains the RP4-specific Mob site in pBR325 and normally is subject to suicide in *Bradyrhizobium* strains. However, when *Bradyrhizobium* strains that contain the broad-host-range plasmid RP4 were used as the recipient, a single crossover event occurred, forming a cointegrate plas-

mid with the inserted region maintaining its integrity (23). When the clones pIM14 and pIM8 were mobilized into *B. japonicum* J1B7 Rif that contained RP4-4, the clone pIM14 conferred the conversion activity on the recipient (Fig. 1). The insert fragment of pIM14 was then digested with *Cla*I, and the 6.5-kb *Cla*I fragment was cloned into pSUP202 to construct pIM6, since *Cla*I did not cut the insert fragment of pRBj7 (Fig. 1). Mobilization of the clone pIM6 resulted in the conversion activity-positive strain, as expected. A restriction map of pIM6 was constructed (Fig. 1). pIM61 and pIM62 were then constructed as described in Materials and Methods. The clone pIM61 conferred the conversion activity on the recipient, while the clone pIM62 did not. The results indicate that the 3.5-kb *Xho*I-*Cla*I fragment from pIM61 contains the gene that confers the conversion activity on *B. japonicum* J1B7 Rif.

Tn*5* mapping. We used Tn*5* mutagenesis to map the gene that confers the conversion activity on *B. japonicum* J1B7 Rif. The Tn*5* insertions in the gene that confers the conversion activity on the recipient were presumably located in the DNA region common to pIM14 and pIM6. To determine whether this supposition was true, pIM14 and pIM6 were mutagenized with Tn*5*. Since pSUP202 is a multicopy plasmid, we used Tn*5* mutagenesis by the method of Simon (23). Plasmids containing Tn*5* insertions in the insert fragments of pIM14 and pIM6 were selected (Fig. 1), and each clone was mobilized into *B. japonicum* J1B7 Rif that harbored RP4-4. The resultant strains were examined for the conversion activity. Plasmids containing Tn*5* insertions C3, C5, C13, and H43 did not confer the conversion activity on the recipient, while other Tn*5* insertions maintained the ability to confer the conversion activity on the recipient. The data indicate that the DNA region that contains the gene that conferred the ability to convert NAM to NAA on the recipient was defined by the Tn*5* insertions that were about 2.3 kb in size, the distance between insertions H17 and H86. In addition, the data suggest that the gene consists of a simple transcriptional unit.

Cloning and expression of indole-3-acetamide hydrolase in *E. coli*. To determine whether the above-mentioned cloning region contains the gene that encodes the enzyme that converts NAM to NAA or whether it contains only a locus that regulates the conversion activity, we cloned the region downstream from the *lacZ* promoter/operator region and examined its expression in *E. coli*. To clone the 3.5-kb *Xho*I-*Cla*I fragment from pIM61 in both orientations with respect to the *lacZ* promoter/operator region, we constructed the plasmids pSK16 and pKS16, as described in Materials and Methods (Fig. 3). We examined the conversion of NAM to NAA by liquid culture-grown cells of *E. coli* JM109 transformed with pSK16 or pKS16. The conversion activity was detected in the case of *E. coli* JM109 cells that harbored the plasmid pSK16 (Fig. 3). The data indicate that this region contains the gene that encodes the enzyme that converts NAM to NAA and, furthermore, that the direction of transcription of the gene was from right to left (as indicated in Fig. 3). To define the 5′ region of the gene in more detail, deletion mutants were constructed by using exonuclease III and mung bean nuclease, as described in Materials and Methods. The results of the deletion mutagenesis are summarized in Fig. 3. Although *E. coli* JM109 harboring pSK161, pSK162, or pSK163 was able to convert NAM to NAA, *E. coli* cells harboring pSK164 or pSK165 were not. The data indicate that the presumed 5′ end of the gene is located adjacent to the *lacZ* promoter/operator

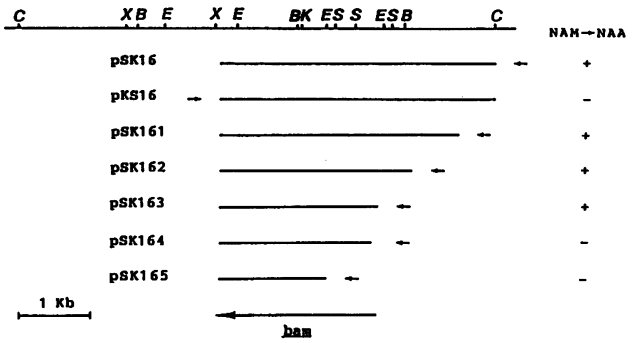

FIG. 3. Deletion mutagenesis analysis of the *bam* gene region of *B. japonicum* DNA. Plasmid pSK16 was deleted by the unidirectional deletion procedure with exonuclease III and mung bean nuclease, as described in Materials and Methods. Expression of the enzyme that converts NAM to NAA in *E. coli* transformants is represented as follows: +, detectable; −, undetectable. The arrow indicates the direction of the *lacZ* promoter/operator region of the *E. coli* plasmid. Restriction sites are shown as represented in Fig. 1.

region between pSK163 and pSK164, and the gene was determined by deletion mutagenesis to be about 2.1 kb long.

To prove that the gene coding for the enzyme that converts NAM to NAA is indeed the gene coding for indole-3-acetamide hydrolase, which is involved in the biosynthesis of IAA, we examined the indole-3-acetamide hydrolase activity of liquid culture-grown cells of *E. coli* JM109 harboring the recombinant plasmids (Fig. 4). The conversion of IAM to IAA was assayed on the basis of the accumulation of IAA in the medium after incubation with IAM. The conversion of IAM to IAA was detected in the transformed *E. coli* cells, while wild-type *E. coli* JM109 showed no conversion activity (Fig. 4). The peak corresponding to authentic IAA has been previously identified by gas chromatographic-mass spectrometric analysis (22). The results indicate that the gene, designated by us as the *bam* gene and which codes for indole-3-acetamide hydrolase, had indeed been cloned, since the IAA biosynthetic pathway has not been reported to occur in *E. coli*.

Similarity of the *bam* gene nucleotide sequences to those of the *iaaH* and *tms-2* genes. Yamada et al. (33) found a high degree of homology between the central regions of the putative products of the *iaaH* gene from *P. savastanoi* and the *tms-2* gene from *A. tumefaciens*. Thus, we performed

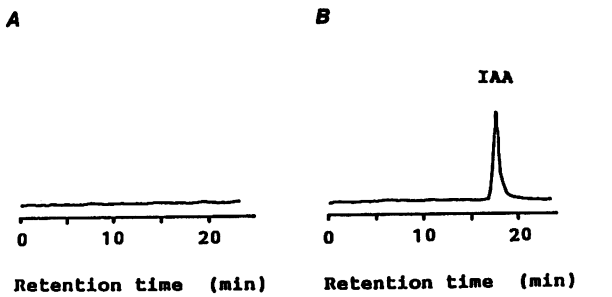

FIG. 4. High-performance liquid chromatographic profile of the IAA fraction of liquid cultures of wild-type *E. coli* JM109 (A) and of *E. coli* JM109 harboring the recombinant plasmid pSK162 (B). Chromatograms were made on Nucleosil 5N(CH_3)$_2$ (length, 100 mm; internal diameter, 6 mm). The column was eluted with 35% acetonitrile in 0.5% acetic acid at a flow rate of 1.5 ml/min. The effluents were monitored by using a spectrofluorometer (exitation, 280 nm; emission, 350 nm).

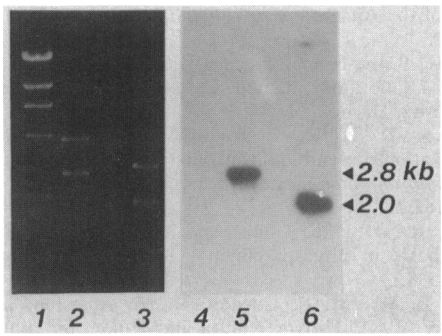

FIG. 5. Hybridization of the ^{32}P-labeled 0.36-kb *Eco*RI-*Kpn*I fragment of the *bam* gene to a Southern blot of *Eco*RI-digested pCP3 carrying the gene *iaaH* from *P. savastanoi* and to that of *Hin*dIII-digested pSP64tms2 carrying the gene *tms-2*. Lanes: 1 and 4, λ DNA digested with *Hin*dIII; 2 and 5, pCP3 digested with *Eco*RI; 3 and 6, pSP64tms2 digested with *Hin*dIII. Lanes 1, 2, and 3 show the results of agarose gel electrophoresis of restriction endonuclease-digested DNAs. Lanes 4, 5, and 6 are Southern blots of DNA probed with the 0.36-kb *Eco*RI-*Kpn*I fragment of the *bam* gene.

Southern hybridization experiments with the central region of the *bam* gene as a probe to investigate the similarity of the *bam* gene to the *iaaH* and *tms-2* genes.

At first, plasmid pCP3 carrying the *iaaM* and *iaaH* genes was digested with *Eco*RI and plasmid pSP64tms2 carrying the *tms-2* gene was digested with *Hin*dIII, and resultant DNAs were separated by electrophoresis on a 0.8% agarose gel and transferred to a nitrocellulose filter (26). pSP64tms2 is plasmid pSP64 that contains a 2.0-kb *Hin*dIII fragment bearing the *tms-2* gene from pTB6HX (Y. Machida, University of Nagoya, personal communication). When Southern blots that contained restriction endonuclease-digested DNAs were hybridized with the ^{32}P-labeled 0.36-kb *Eco*RI-*Kpn*I fragment of the *bam* gene, the 2.8-kb *Eco*RI fragment of pCP3 and the 2.0-kb *Hin*dIII fragment of pSP64tms2 were hybridized under high-stringency washing conditions (Fig. 5); the other fragments were not hybridized. This result indicates that a high degree of similarity is present among the central regions of the nucleotide sequences of these three genes.

DISCUSSION

We have previously suggested the presence of the IAM pathway in free-living cultures of *B. japonicum* (22) and detected the enzymatic conversion of NAM to NAA in soybean root nodules (M. Sekine, unpublished results). The results suggested that the IAM pathway might also operate in the bacteroidal state of *B. japonicum*, since no such activity has been detected in soybean root and since the natural occurrence of IAM in plants, reported previously, is doubtful (21). The identification and isolation of genes involved in the IAM pathway will allow detailed analysis of the involvement of the IAM pathway in the symbiotic relationship between *B. japonicum* and the soybean plant.

We have attempted unsuccessfully to isolate the genes from *B. japonicum* that are involved in the IAM pathway by using the *tms-1* and *tms-2* sequences from *A. tumefaciens* as hybridization probes. In our previous paper (22), we demonstrated that the indole-3-acetamide hydrolase activity can be replaced by an activity that generates NAA from NAM, an analog of IAM. Therefore, we have attempted to isolate a gene coding for indole-3-acetamide hydrolase by using the ability to convert NAM to NAA as a biological marker. We constructed a cosmid bank of *B. japonicum* J1063, which possesses the enzyme that converts NAM to NAA. We mobilized the appropriate DNA fragment into the defective strain, *B. japonicum* J1B7 Rif^r, to screen for the clone(s) which had regained the hydrolase activity.

We cloned the gene into the *E. coli* plasmid Bluescript SK M13$^+$ and detected the enzymatic activity that converts NAM to NAA, as well as a high level of expression of indole-3-acetamide hydrolase activity in *E. coli* transformants (Fig. 4). To our knowledge, this is the first report of the cloning of a gene involved in the biosynthesis of IAA in *Rhizobium* and *Bradyrhizobium* strains. When the cloned insert was oriented in the opposite direction in the vector, indole-3-acetamide hydrolase was not detected in *E. coli* transformants (Fig. 3). A very low level of activity of indole-3-acetamide hydrolase was detected in *E. coli* transformants cultured without IPTG. However, expression increased about 20- to 30-fold upon the addition of IPTG to liquid cultures (data not shown). Therefore, the efficient expression in *E. coli* of the *bam* gene for indole-3-acetamide hydrolase from *B. japonicum* requires the presence of the *lacZ* promoter.

Recently, differences in the transcription-translation of *nod* genes from *R. meliloti* were observed when expression of proteins was compared in cell-free systems derived from *Rhizobium meliloti* and *E. coli* (7).

The IAM pathway is well studied in *P. savastanoi* and in *A. tumefaciens*. The genes for tryptophan 2-monooxygenase (*iaaM*) and indole-3-acetamide hydrolase (*iaaH*) are organized in an operon, and the only bacterium in which they function is *P. savastanoi* (4, 5), whereas the comparable genes in *A. tumefaciens*, *tms-1*, and *tms-2*, are monocistronic and function only in plants (11, 15, 19, 21, 28–30). Recently, significant homology between the gene *iaaM* and the gene *tms-1* and between the gene *iaaH* and the gene *tms-2* was found in terms of the nucleotide sequences and deduced amino acid sequences (33). Furthermore, it has been proposed that the genes for the IAM pathway of *P. savastanoi* and *A. tumefaciens* originated from common ancestral genes (11, 33). In the case of *B. japonicum*, the genes coding for indole-3-acetamide hydrolase should be active in both the bacterial (free-living) and the bacteroidal (symbiotic) state and should, most likely, be different from a gene for tryptophan 2-monooxygenase. In fact, we did not find any similarity between the nucleotide sequence of the upstream and/or downstream region of the *bam* gene and that of the tryptophan 2-monooxygenase genes, *iaaM* and *tms-1* (data not shown). We do not know the location of the tryptophan 2-monooxygenase gene in *B. japonicum*, but we are investigating this gene.

As shown in Fig. 5, we have found a high degree of similarity among the central regions of the nucleotide sequences of the *bam*, *iaaH*, and *tms-2* genes. This result agrees with the result of the nucleotide sequence of the *bam* gene; that is, the 0.36-kb *Eco*RI-*Kpn*I fragment, which used a hybridization probe, contains the core regions of the *bam* gene that are highly homologous with the central regions of the putative products of the *iaaH* and *tms-2* genes (data not shown). These results suggest that these three genes originated from a common ancestral gene.

ACKNOWLEDGMENTS

We thank C. Sasakawa for providing *E. coli* DB1572 and for advice on Tn*5* mutagenesis procedures. We thank T. Yamada and Y. Machida for providing pCP3 and pTB6HX, respectively. We

thank A. Pühler and F. M. Ausubel for providing pSUP202 and RP4-4 and pRK290 and pLAFR1, respectively.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science and Culture.

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