Cloning and Expression in *Escherichia coli* of the *Azospirillum brasilense* Sp7 Gene Encoding Ampicillin Resistance

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The *Azospirillum brasilense* ATCC 29145 gene coding for β -lactamase was cloned in *Escherichia coli*. The gene was expressed in *E. coli* from its own promoter as a 30-kilodalton protein, conferring resistance to high levels of β -lactam antibiotics. The DNA sequence containing the β -lactamase gene was found to be highly amplified in the *Azospirillum* genome, scattered in the chromosomal as well as in the plasmidic DNA.

Bacteria of the genus *Azospirillum* are free-living diazotrophs that can readily be isolated from the rhizosphere and roots of forage and grain grasses (7). Since the identification of these bacteria, their economic potential as inoculants for cereal crops has stimulated intensive research in many laboratories to identify genes involved in nitrogen fixation and plant interaction by *Azospirillum* spp. (for reviews, see references 8 and 19). There is still a need for the expansion of suitable genetic tools for the analysis of the structure, organization, and expression of the *Azospirillum* genome. Little is known about gene-controlling elements in *Azospirillum* species. The isolation of a strong promoter acting in a wide range of *Azospirillum* isolates would be very useful for the construction of expression vectors. For this purpose, we chose to clone the gene encoding ampicillin resistance. Most if not all *Azospirillum* isolates are resistant to high levels (400 μ g/ml) of ampicillin, and the resistance is believed to be due to β -lactamase activity (10). In this paper we report on the molecular cloning of a DNA fragment encoding *A. brasilense* ATCC 29145 β -lactamase.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1.

Media and growth conditions. *Escherichia coli* strains were grown in LB broth or agar, supplemented with the appropriate antibiotics. Concentrations of antibiotics were 100 μ g of ampicillin per ml, 25 μ g of kanamycin per ml, and 10 μ g of tetracycline per ml, unless specified otherwise. *Azospirillum* strains were grown in yeast extract-peptone (YEP) broth or agar at 30°C.

Enzymes and chemicals. All restriction endonucleases and DNA-modifying enzymes were purchased from Amersham U.K. and used as specified by the manufacturer. [α - 32 P]dCTP (specific activity, >400 Ci/mmol) and L-[35 S]methionine (specific activity, >1,000 Ci/mmol) were purchased from Amersham U.K.

DNA isolation. *Azospirillum* total DNA was isolated as described by Meade et al. (17). Lysates of *Azospirillum* strains were obtained by the method of Kado and Liu (14). Plasmid DNA from *E. coli* was prepared by using the cleared-lysate procedure (2). When used as a hybridization probe, plasmid DNA was further purified by centrifugation through cesium chloride-ethidium bromide density gradi-

ents. DNA fragments from agarose gels were isolated by electroelution.

Molecular cloning. For the construction of the cosmid library of *A. brasilense* ATCC 29145, total DNA was partially digested with *Eco*RI. DNA was size fractionated by centrifugation as described by Friedman et al. (11). Fractions containing DNA fragments of 20 to 30 kilobase pairs (kb) were pooled and concentrated. This DNA was ligated to *Eco*RI-cleaved pLAFR1 at a concentration of approximately 200 μ g of *A. brasilense* DNA per ml and about 40 μ g of vector DNA per ml. The ligated mix was packaged in bacteriophage lambda heads with the packaging kit of Amersham and was used to infect *E. coli* HB101 cells.

DNA hybridization. DNA hybridizations were conducted overnight on Hybond-N (Amersham U.K.) nylon membranes. Southern-blotted DNA hybridizations were done as described by Silhavy et al. (24). [α - 32 P]dCTP-labeled probes (specific activity, >5 $\times$ 10⁷ cpm/ μ g of DNA) were obtained by using either the nick translation kit or the Multiprime labeling kit of Amersham U.K. Filters were autoradiographed at -80°C by using Fuji RX films and intensifying screens (Kyokko special).

Enzymogram for β -lactamase activity. The semiquantitative method, based on decolorization of the iodine-iodide-starch complex, of Labia and Barthélémy (15) was used. Cells grown in the absence or presence of carbenicillin were washed several times in phosphate buffer (pH 7.0) and suspended in phosphate buffer to their original volume. Samples of 10 μ l were mixed with 50 μ g of penicillin G, spotted on iodine-iodide-starch plates, and incubated for 60 min at 4°C.

Minicell labeling and polyacrylamide gel electrophoresis. Plasmids were introduced by transformation into *E. coli* DS410. Minicells were isolated by the method of Reeve (21), with slight modifications (A. Lejeune, unpublished data). Minicells were labeled with [35 S]methionine as described previously (22). Polypeptides were separated on sodium dodecyl sulfate-polyacrylamide gels as described previously (16) and detected by fluorography.

RESULTS

Cloning of β -lactamase gene. *A. brasilense* ATCC 29145 DNA was partially digested with *Eco*RI and size fractionated by sucrose gradient centrifugation. Fractions containing DNA fragments between 20 and 30 kb in size were ligated to *Eco*RI-digested pLAFR1 vector DNA and packaged in vitro as outlined in Materials and Methods. *E. coli* HB101 cells

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Source or reference
<i>E. coli</i>		
HB101	F ⁻ <i>hsdS20</i> (<i>r_B</i> ⁻ <i>m_B</i> ⁻) <i>recA13 ara-14 proA2</i> <i>lacY1 galK2 rpsL20</i> (Sm ^r) <i>xyl-5 mtl-1 supE44</i> λ ⁻	4
HB101 <i>chrX</i> ::Tn5	<i>E. coli</i> Tn5 donor strain	C. Koncz
JC5466	<i>E. coli</i> Tn5-Mob donor strain	P. Mazodier
GV1000	Rifampin-resistant derivative of C600	13
DS410	Minicell-producing strain	A. Lejeune
ABG4-1	MG1555 carrying chromosomal Tn10 dCam insertion, Cm ^r	K. Michiels
<i>A. brasilense</i>		
Sp7	Ap ^r	ATCC 29145
Sp107	Ap ^r	J. Döbereiner
RO7	Ap ^r	K. Vlassak
Sp245	Ap ^r	J. Döbereiner
Sp246	Ap ^r	J. Döbereiner
<i>A. lipoferum</i>		
SpBr17	Ap ^r	ATCC 29709
Sp59b	Ap ^r	W. Klingmüller
Plasmids		
pLAFR1	pRK290, containing the λ <i>cos</i> site, Tc ^r	11
pRK600	ColE1 replicon with the <i>tra</i> functions of RK2, <i>npt</i> ::Tn9	6
pBR322	Ap ^r Tc ^r	3
pBR322-1	Ap ^s Tc ^r , <i>Dra</i> I deletion derivative of pBR322	This work
pGV462	High-copy-number derivative of pBR322	25

were transfected with package mix and plated on LB agar containing 10 μ g of tetracycline per ml. Approximately 1,000 *E. coli* clones were replica plated on LB agar containing tetracycline and 100 μ g of ampicillin per ml. One particular clone was resistant to ampicillin. Plasmid DNA of this clone was extracted, digested with *Eco*RI or *Sal*I or both, and characterized by agarose gel electrophoresis (Fig. 1). The cosmid clone, hereafter designated as pAp100, contains approximately 20 kb of insert DNA. pAp100 DNA was transformed into *E. coli* HB101 *chrX*::Tn5. All the tetracycline-resistant transformants were also resistant to ampicillin, indicating that the ampicillin resistance is encoded by the plasmid DNA. Several independent transformants were crossed with *E. coli* GV1000 in a triparental mating with *E. coli* HB101(pRK600) as a helper. Transconjugants were selected on LB medium, supplemented with tetracycline (10 μ g/ml) for the marker of the pLAFR1 replicon, kanamycin (25 μ g/ml) for the Tn5 marker, and rifampin (150 μ g/ml) for the GV1000 chromosomal marker. Transconjugants were subjected to single-colony purification and were replica plated on LB agar supplemented with ampicillin (100 μ g/ml). Of 885 colonies tested, 17 were sensitive to ampicillin, indicating gene inactivation by Tn5 insertion. This was confirmed by *Sal*I restriction analysis of plasmid DNA of the Km^r Ap^s clones. In all these plasmids, a Tn5 insertion had occurred in the 9.1-kb *Sal*I restriction fragment of pAp100 (Fig. 1).

FIG. 1. Restriction digest of cosmid clone pAp100. Lanes: a, replicative-form DNA of pAp100; b, *Hind*III digest of lambda DNA; c, *Eco*RI digest of pAp100; d, *Sal*I digest of pAp100; e, *Eco*RI-*Sal*I digest of pAp100.

Mapping of the β -lactamase gene. The 9.1-kb *Sal*I restriction fragment of pAp100 was subcloned in pGV462 and in pBR322-1, a *Dra*I deletion derivative of pBR322, lacking almost the entire *bla* gene of pBR322. Both constructs, pGVAp-1 and pBRAp, regardless of the orientation of the insert DNA, conferred ampicillin resistance on the *E. coli* HB101 host. These data suggest that the *A. brasilense* ampicillin resistance gene is located on a 9.1-kb *Sal*I restriction fragment and that its transcription in *E. coli* was initiated from its own promoter. A physical map of the 9.1-kb *Sal*I restriction fragment is shown in Fig. 2A. Subclones of the 9.1-kb *Sal*I restriction fragment that were sufficient to encode ampicillin resistance in *E. coli* are shown in Fig. 2B. The construct pBRAp was subjected to Tn5-mob

FIG. 2. Physical and genetic map of the 9.1-kb *Sal*I fragment of pAp100. (A) Symbols: $\blacktriangle$, positions of Tn5-mob insertions; +, Tn5-mob insertions that do not inactivate the *bla* gene; -, Tn5-mob insertions that inactivate the cloned *bla* gene in *E. coli*. Abbreviations: S, *Sal*I; B, *Bgl*II; P, *Pst*I; E, *Eco*RI. (B) Subclones of the *Sal*I restriction fragment that confer ampicillin resistance on the *E. coli* host.

FIG. 3. Expression of the *A. brasilense bla* gene in *E. coli* DS410 minicells. Proteins were labeled, extracted, and separated on polyacrylamide gels as explained in Methods and Materials. Lanes: a, DS410(pGV462) cells; b, DS410 cells; c, DS410(pGVAp-1) cells; d, DS410(pGVAp-2) cells. kDa, Kilodaltons.

mutagenesis in *E. coli* JC5466. Tn5-*mob*-containing constructs were selected in *E. coli* ABG4-1 and tested for ampicillin sensitivity. The exact site of the Tn5-*mob* insertion was determined by restriction analysis. The results are summarized in Fig. 2A. It can be concluded that the β -lactamase gene spans the sequence from the *Eco*RI restriction site to slightly beyond the *Pst*I restriction site, since an *Eco*RI-*Pst*I subclone did not confer ampicillin resistance on the *E. coli* host.

Characterization of β -lactamase produced by *E. coli* (pGVAp-2). The constructs pGVAp-1 and pGVAp-2 were transformed into the minicell-producing *E. coli* DS410. Plasmid-encoded proteins were extracted and detected as described in Materials and Methods. The results (Fig. 3) show that plasmids pGVAp-1 and pGVAp-2, in comparison with the cloning vector used (pGV462), code for an additional polypeptide of approximately 30 kilodaltons. This polypeptide most probably corresponds to the polypeptide encoded by the ampicillin resistance gene. β -Lactamase production by *E. coli*(pGVAp-2) was confirmed by the enzymogram method as described in Materials and Methods, with penicillin G as the substrate (data not shown). *E. coli* HB101 cells containing either pBRAP or pGVAp-2 are resistant to ampicillin concentrations up to 2 mg/ml. Moreover, an ampicillin-sensitive pBRAP clone containing Tn5-*mob* close to the *Eco*RI site (Fig. 2A) did not encode the 30-kilodalton protein (results not shown).

Organization of the β -lactamase gene in the *Azospirillum* genome. The 0.9-kb *Pst*I-*Eco*RI DNA fragment of pBRAP (Fig. 2) was used as a probe in hybridizations on restricted total and subcloned *A. brasilense* ATCC 29145 DNA (Fig.

FIG. 4. Southern blot hybridization on restricted total DNA of *A. brasilense* Sp7 or Sp245 and cloned DNA of *A. brasilense* Sp7 with a 0.9-kb *Pst*I-*Eco*RI-labeled restriction fragment of *A. brasilense* Sp7, containing the *bla* gene. Lanes: A and B, total DNA of *A. brasilense* Sp7; C, total DNA of *A. brasilense* Sp245; D to F, cloned DNA of *A. brasilense* Sp7 in *E. coli*. Lane A, *Eco*RI digest; lane B, *Pst*I-*Eco*RI digest; lane C, *Pst*I-*Eco*RI digest; lane D, *Pst*I-*Eco*RI digest of pAp100; lane E, *Pst*I-*Eco*RI digest of pBRAP; lane F, *Pst*I-*Eco*RI digest of pGVAp-1.

4). In all subclones tested and the total *A. brasilense* DNA, the probe hybridizes with the same 0.9-kb *Pst*I-*Eco*RI restriction fragment (Fig. 4, lanes B to F). The second hybridizing band in lane E most probably represents homology of the probe with part of the *bla* sequence of pBR322 that was not deleted in pBR322-1. However, it is also evident that this particular DNA sequence is highly amplified in the genome of *A. brasilense* Sp7 and Sp245 (lanes A to C), as well as in the genome of three other *Azospirillum* strains tested, i.e., SpBr17, Sp59b, and R07 (data not shown). This observation raised the question of why we could initially isolate from the cosmid library only one particular clone (pAp100) conferring ampicillin resistance on the *E. coli* host. Therefore, we decided to probe the cosmid library of *A. brasilense* ATCC 29145 DNA with the 0.9-kb restriction fragment by colony hybridizations. Five hybridizing clones were identified. None of these, however, had an ampicillin-resistant phenotype, and the resident cosmid clones did not contain overlapping DNA fragments. This indicates that the tested DNA sequence is scattered in the genome of *A. brasilense* ATCC 29145. All *Azospirillum* strains, like *A. brasilense* ATCC 29145, contain plasmids in various numbers and of various sizes (10). To determine the chromosomal or plasmid localization of the sequences homologous to the 0.9-kb *Pst*I-*Eco*RI restriction fragment, we performed Southern blot hybridization on lysates (14) of various *Azospirillum* strains. For all strains tested, a hybridization signal was detected with the chromosomal fraction (Fig. 5). For strains Sp7 (ATCC 29145), Sp107, Sp246, Sp59b, and SpBr17, a hybridization signal was also observed with plasmid DNA.

DISCUSSION

Despite increasing interest in developing effective *Azospirillum* strains for inoculation of crop plants, little is known about the genetics of these bacteria. Up to now, the only *Azospirillum* gene for which the nucleotide sequence has

FIG. 5. Southern blot hybridization on lysates (14) of various *Azospirillum* strains with a 0.9-kb *Pst*I-*Eco*RI-labeled restriction fragment of *A. brasilense* Sp7, containing the *bla* gene. Lanes: A, Sp7; B, Sp107; C, Sp245; D, Sp246; E, Sp59b; F, RO7; G, SpBr17.

been determined is the *glnA* gene of *A. brasilense* Sp7 (5). The gene is not expressed in *E. coli*, and no significant homology with *E. coli* canonical promoters was found. It is believed that failure of expression occurs at the transcriptional level, since fusion of the coding sequence with a promoter functioning in *E. coli* leads to expression of the *glnA* gene in *E. coli*, despite a very biased codon usage.

Evidence for expression of *A. brasilense* genes in heterologous systems, as reported here for the *bla* gene, has also been obtained by Bazzicalupo et al. (1). They demonstrated the expression of amino-acid-biosynthetic genes in *E. coli*. Recently, we were able to demonstrate the expression of *Azospirillum* genes, involved in plant interaction, in *Rhizobium meliloti* (18). A nucleotide sequence analysis of these genes is in progress (C. Verreth, unpublished data; M. Bazzicalupo, personal communication), and it will be of interest to compare the 5' upstream sequences of these genes with the published sequence of the *glnA* gene.

The data on the occurrence, duplication, and expression of an ampicillin resistance gene in *A. brasilense* spp. raise several points for further analysis. Since the report of Sapienza and Doolittle (23) on DNA reiteration as a common feature of the genome of archaeobacteria, other reports have suggested DNA reiteration as a common genomic feature in the eubacteria *Rhizobium* and *Agrobacterium* spp. (9, 20). The nature of some of the repeated elements has been established for various *Rhizobium* species: *nif* genes, regulatory regions, an early nodulation gene, insertion sequences. For *Azospirillum* spp. reported in this paper, it is tempting to speculate that the observed DNA reiteration represents a mobile genetic element. A number of transposons that determine ampicillin resistance by a β -lactamase gene have been identified (12). In fact, preliminary hybridization studies suggest homology between the *bla* gene of Tn3 and the cloned *bla* gene of *A. brasilense* Sp7 (Fig. 4). However, we could not detect DNA homology between the pAp100 DNA and a DNA fragment of Tn3 containing the transposase. This observation requires further exploration. Finally, it will be of interest to explain the observation that only one particular copy of the *bla* gene of *A. brasilense* is expressed in *E. coli* and to determine the functionality of each copy in *Azospirillum* species.

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