

New Mobilizable Vectors Suitable for Gene Replacement in Gram-Negative Bacteria and Their Use in Mapping of the 3' End of the *Xanthomonas campestris* pv. *campestris* *gum* Operon

FEDERICO KATZEN,¹ ANKE BECKER,² M. VERÓNICA IELMINI,¹
CRISTIAN G. ODDO,¹ AND LUIS IELPI^{1*}

Instituto de Investigaciones Bioquímicas Fundación Campomar, Facultad de Ciencias Exactas y Naturales, UBA, and CONICET, 1405 Buenos Aires, Argentina,¹ and Lehrstuhl für Genetik, Universität Bielefeld, D-33501 Bielefeld, Germany²

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We describe useful vectors to select double-crossover events directly in site-directed marker exchange mutagenesis in gram-negative bacteria. These vectors contain the *gusA* marker gene, providing colorimetric screens to identify bacteria harboring those sequences. The applicability of these vectors was shown by mapping the 3' end of the *Xanthomonas campestris* *gum* operon, involved in biosynthesis of xanthan.

Gene disruption and marker exchange techniques are well-established tools for genetic engineering of bacteria. Site-directed marker exchange methods include several steps: (i) cloning of a gene region into a suicide vector, (ii) disruption of the cloned gene by insertion of a transposon or an interposon cassette, (iii) mobilization of the hybrid plasmid to the recipient strain, (iv) selection of recombinant strains, and (v) identification of double-recombinant strains. In many cases the last step involves tedious work, since the ratio of double-homologous-crossover to single-crossover events can be very low, depending on the organism used. This difficulty can occasionally be overcome by incorporating a copy of the *sacB* gene coding for the levansucrase enzyme (16–18), whose expression in a sucrose-containing medium leads to a lethal phenotype in some organisms. Unfortunately, in many gram-negative bacteria the expression of this gene in a sucrose-containing medium is not lethal or leads to the growth of small colonies in prolonged incubations (18). To solve this problem, we developed two plasmid vectors containing a marker gene that enables us to discern between single- and double-crossover events without any experimental step.

The *gusA* gene, originally identified in *Escherichia coli* (11), is a widespread marker mainly used to study plant gene expression. It encodes the β -glucuronidase enzyme (GUS). The advantages of GUS over other reporter systems include the robustness of the enzyme and the simplicity of the assays. Compared to the products of other reporter genes, GUS is a very stable gene product. This enzyme accumulates over time, and minute quantities of GUS activity can be accurately measured (12). A key advantage of GUS is the generalized absence of this activity within the taxon Eubacteria (25), with the exception of *E. coli* and some species of *Shigella*, *Streptococcus*, *Staphylococcus*, *Corynebacteria*, and *Clostridium*. The incorporation of the *gusA* gene into the mobilizable suicide vectors pK18mob and pK19mob facilitates discerning between gene

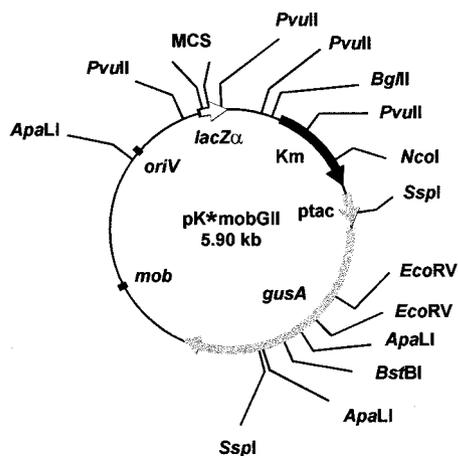
replacement and plasmid integration by simply observing the color of the colonies.

Construction of pKmobGII vectors. A restriction map of the resulting pKmobGII vectors and the nucleotide sequence of their multiple cloning site (MCS) are shown in Fig. 1. For the construction of the vectors, the *gusA* gene together with the polyadenylation signal from the nopaline synthase gene (NOS-ter) from the low-copy-number plasmid pBII101 (GenBank accession no. U12639 [12]) were cloned between the *Hind*III and *Eco*RI sites of pUC19, giving rise to pDNA4. The *tac* promoter (GenBank accession no. K01728) was released from plasmid pKK223-3 (5) by digestion with *Eco*RI and *Bam*HI, and the *Eco*RI end was filled in. This fragment was cloned into pDNA4, which was previously digested with *Bam*HI and *Sma*I, originating pTACDNA4. The final plasmids were constructed by cloning the *Bam*HI-*Sac*I fragment, containing the *gusA* gene from pTACDNA4, into *Bst*BI-digested pK18mob and pK19mob plasmids (GenBank accession no. AF012346 [17]) upon filling in and removing the 3' and 5' recessed ends. The additional *Bam*HI site regenerated at the *Bam*HI-*Bst*BI junction was removed by filling in with the Klenow enzyme. These high-copy-number plasmids are 5.9 kb in length and contain the origin of replication of pUC vectors; hence, their host range is restricted to *E. coli* and a few other species of the *Enterobacteriaceae* family. The presence of an origin of transfer (*mobRK2*) enables their transference by conjugation into a wide range of bacteria (17). They also carry the Tn5 kanamycin resistance gene. Selection for this antibiotic resistance does not lead to the formation of satellite colonies and results in a high yield of DNA from standard plasmid preparations (15). The pUC multiple cloning site located within the gene encoding the LacZ α peptide enables the application of these vectors as standard cloning vectors.

These plasmids were successfully tested in *Xanthomonas campestris* pv. *campestris* FC2 (14), *Rhizobium meliloti* 1021 (1), and *Agrobacterium tumefaciens* A348 (7). For this purpose, DNA fragments of *X. campestris*, *A. tumefaciens*, and *R. meliloti* were subcloned into pK19mobGII and the resulting hybrid plasmids were transferred to their correspondent wild-type host from the broad-host-range mobilizing strain *E. coli* S17-1, as described previously (19). Integration of hybrid plasmids into

* Corresponding author. Mailing address: Instituto de Investigaciones Bioquímicas Fundación Campomar, Patricias Argentinas 435, 1405 Buenos Aires, Argentina. Phone: 54(1) 863-4011/19. Fax: 54(1) 865-2246. E-mail: LIELPI@iib.uba.ar.

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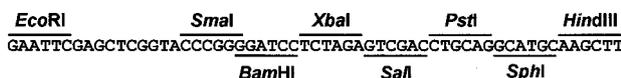


FIG. 1. (A) Physical map of pKmobGII vectors. The asterisk symbolizes the plasmid label 18 or 19. (B) Nucleotide sequence of the MCS. Unique restriction sites for cloning purposes are indicated. The enzyme recognition sites and the orientation of the MCS correspond to those in plasmids pK18mob and pK19mob. Km, kanamycin resistance gene (black arrow); *mob*, origin of transfer; *oriV*, origin for vegetative replication; *lacZα*, *lac* fragment for α -complementation (open arrow); *ptac*, *tac* promoter (small gray arrow); *gusA*, β -glucuronidase gene (large gray arrow).

their correspondent host genomes was selected by the vector-encoded antibiotic resistance. Cells carrying pKmobGII sequences integrated into their chromosomes can be easily distinguished from those lacking these sequences in plates containing 50 mg of 5-bromo-4-chloro-3-indoxyl- β -D-glucuronide (X-gluc)/liter or 25 mg of 5-bromo-6-chloro-3-indoxyl- β -D-glucuronide (magenta-gluc)/liter. Phenotypes of *X. campestris*, *R. meliloti*, and *A. tumefaciens* cells carrying or lacking pKmobGII sequences are shown in Fig. 2. GUS activities were assayed by following a protocol originally designed for plants (10). Relative units of GUS for a strain were calculated by using the following formula: (picomoles of methyl-umbelliferone \times reaction volume [in milliliters]) / (time [in minutes] \times OD₆₀₀ of culture \times cell culture [in milliliters]), where OD₆₀₀ is the optical density at 600 nm.

Although GUS activity that results from the expression of pKmobGII sequences varies among different hosts (Fig. 2), a correlation between GUS activity and cell color development was not observed. Whereas blue colonies of *A. tumefaciens* and *R. meliloti* strains could be observed 2 days after plating, *X. campestris* cells harboring a copy of pKmobGII needed to be stored at 4°C for a week to become green (Fig. 2). This might be due to differences in the cell wall composition that could make *X. campestris* cells more impermeable to chromogenic substrates.

The only requisite for the usefulness of these vectors is that the synthesized GUS protein should become accessible to its substrate (X-gluc). There is no need for any specific sugar consumption by the cell for color development. The catalysis of

X-gluc might proceed either in the cytoplasm, if the substrate gains access to it, or outside the cell, if the enzyme is released by spontaneous lysis. To test whether the presence of the glucuronide-specific permease gene *gusB* (11) facilitated color development, this gene was cloned into the broad-host-range vector pRK293 and introduced into a *X. campestris* mutant with pKmobGII sequences integrated into its chromosome. The presence of the *gusB*-containing plasmid did not shorten the time in which cells developed a green color.

Mapping of the 3' end of the *gum* operon in *X. campestris*. The plant-pathogenic bacterium *X. campestris* produces an acidic exopolysaccharide termed xanthan gum, which is composed of D-glucosyl, D-mannosyl, and D-glucuronyl acid residues in a molar ratio of 2:2:1 and variable proportions of O-acetyl and pyruvyl residues (9, 21). Because of its physical properties, xanthan is widely used as a thickener or viscosifier in both the food and nonfood industries (reviewed in reference 3). The biosynthesis of xanthan mainly consists of a stepwise assembly and decoration of pentasaccharide subunits attached to a polyprenol phosphate carrier, which are subsequently polymerized and exported (8). The enzymes required for these processes are encoded by a 16-kb genome region named *xpsI*, or *gum* (14). This region is composed of 12 genes designated *gumB* to *gumM* (GenBank accession no. U22511). Transcription of the *X. campestris* *gum* gene cluster was proved to be directed by a promoter located upstream of the first gene, *gumB* (13), but the 3' end of the operon has not been mapped.

In order to assess whether this operon continues downstream of *gumM*, we sequenced a 3.6-kb DNA fragment adjacent to the *gum* region contained in the plasmid pJC372 (J. M. Cleary, Kelco Biopolymers Group of Monsanto Company) (Fig. 3A). This plasmid was obtained from a *X. campestris* genomic library constructed by cloning total DNA digested with *SacI* into pJC106, a derivative of the broad-host-range vector RSF1010. DNA sequences were obtained from overlapping nested deletion clones generated by exonuclease III digestion and processed by using an ALFexpress DNA sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. The nucleotide and amino acid sequences were analyzed by using the MacVector sequence analysis software (Oxford Molecular Ltd., United Kingdom). The amino acid sequences deduced from the nucleotide sequence were compared to the GenBank database with the gapped BLAST algorithm (2). A coding region analysis, using a *X. campestris* codon usage table, suggested the presence of three open reading frames, which were named, from 5' to 3', ORF14, ORF15, and ORF16 (Fig. 3B). While a comparison of the deduced amino acid sequences of ORF14 and ORF16 revealed no homologies in the GenBank database, the deduced sequence of ORF15 was found to be homologous to those of a large family of 3-ketoacyl-acyl carrier proteins involved in the biosynthetic pathway of fatty acids (24).

To test whether ORF14 is cotranscribed with *gumM*, we employed a method consisting of the application of *lacZ* transcriptional fusions combined with plasmid integration (13). A promoterless *lacZ-aacC1* interposon (4) was inserted into the unique *Bam*HI site of the *SmaI-ClaI* fragment (nucleotides 14056 [GenBank accession no. U22511] and 2071 [GenBank accession no. U70053], respectively), which was previously subcloned into pK19mobGII. The hybrid plasmid was transferred from the broad-host-range mobilizing strain *E. coli* S17-1 (21) to wild-type *X. campestris* FC2 (14), as described previously (19). Exconjugants were selected in agar medium containing gentamicin, rifampin, and X-gluc. Yellow colonies appeared with a frequency of 10^{-4} and were sensitive to kanamycin. Correct gene replacement was verified by Southern hybridiza-

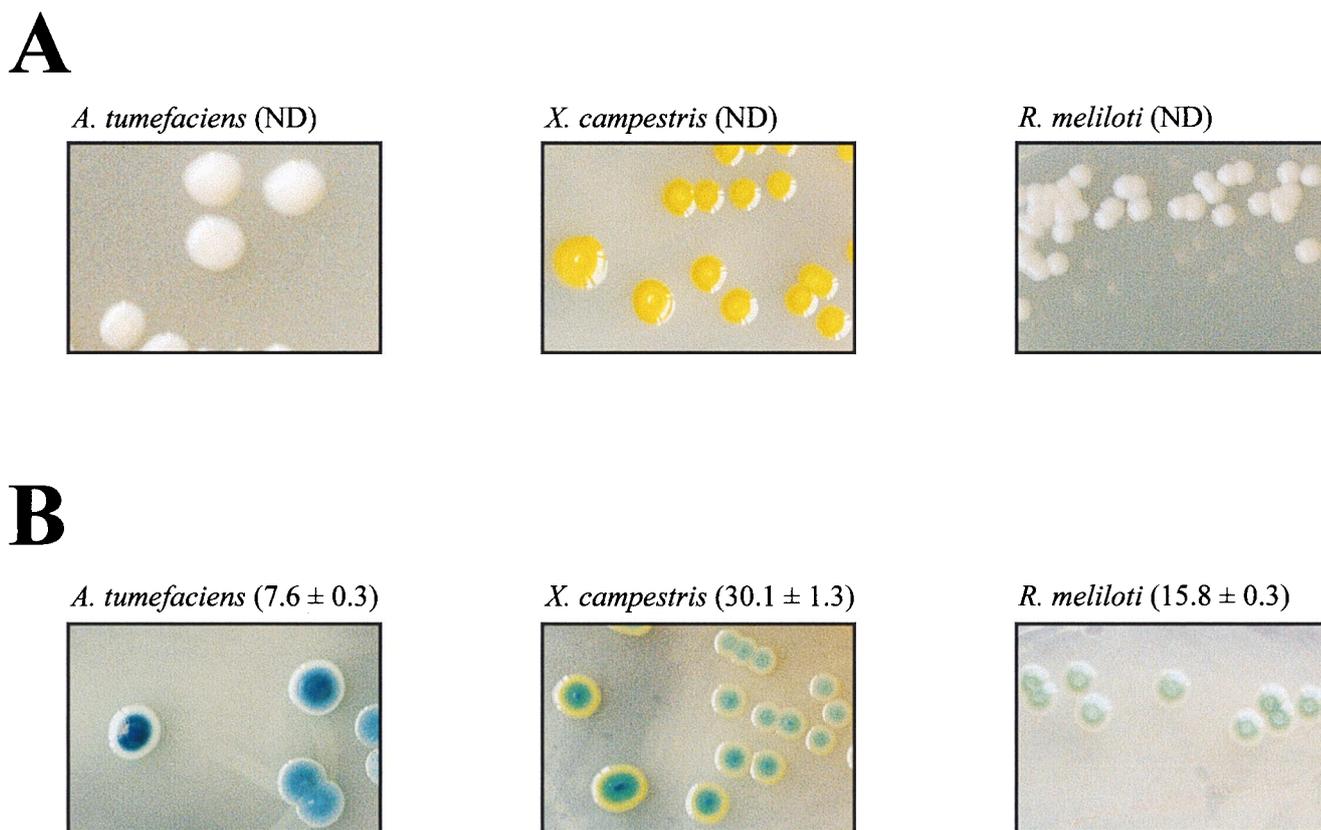


FIG. 2. Phenotypes of different strains grown on X-gluc-containing medium. (A) *X. campestris* FC2, *R. meliloti* Rm1021, and *A. tumefaciens* A348 wild-type cells grown for 2 days at 28°C in tryptone-yeast plates (22) containing 50 µg of X-gluc/ml. (B) The same cells harboring pK19mobGII sequences integrated into their genomes were grown as described for panel A in the presence of 50 µg of kanamycin/ml. The GUS activity of each strain is indicated in parentheses. The GUS activities were assayed by employing five independent samples. ND, not detected. The photographs of *X. campestris* cells were taken after storing the plates for 7 days at 4°C.

tion. The strain mutated in ORF14 (XcORF14) produced normal amounts of xanthan, as judged by precipitation of the polymer from the broth as previously described (6) (not shown). Also, the biochemical characterization of their lipid-linked intermediates, following established protocols (14), revealed that XcORF14 is capable of both synthesizing and decorating the

xanthan lipid-linked repeating unit (not shown). A second mutant in ORF14 was built up by plasmid integration. An internal fragment of ORF14 (nucleotides 763 to 1170; GenBank accession no. U70053) was cloned into the mobilizable suicide vector pK18mob. The resulting plasmid was transferred to *X. campestris* FC2, and the integration of the hybrid plasmid into the

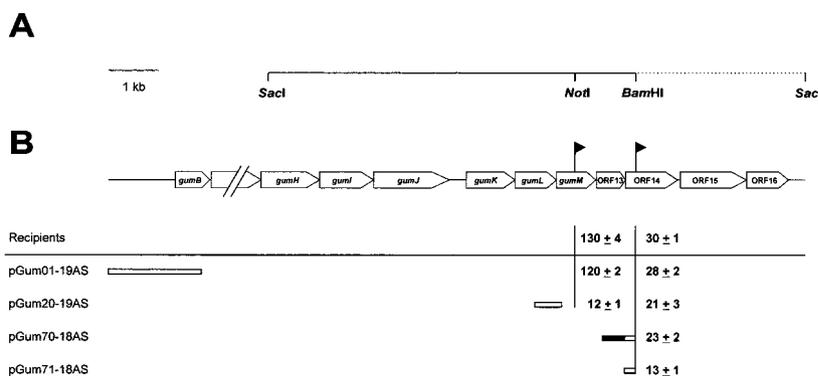


FIG. 3. Mapping of the 3' end of the *gum* operon. (A) Physical map of the plasmid pJC372. DNA sequences that overlap the *gum* region (GenBank accession no. U22511) are indicated by a solid line. The dotted line represents the nucleotide sequence of a 3.6-kb DNA fragment determined in this work (GenBank accession no. U70053). The relevant restriction sites are indicated. (B) Genetic map of the cosmid pJC372. The position and orientation of *lacZ* transcriptional fusions are indicated by solid flags. The relative activities (\pm standard deviations) of *lacZ* transcriptional fusions are given in β -Gal units (Miller units) above the line. Below the line, the restriction fragments used for integration mutagenesis are presented. A potential promoter is indicated by a solid box. The relative β -Gal activities (\pm standard deviations) of the *lacZ* transcriptional fusions located downstream from the vector integration sites are listed; the activities were calculated from five independent measurements.

X. campestris genome was selected by the vector-encoded antibiotic resistance and verified by Southern hybridization. The phenotype of this mutant was identical to that of XcORF14, reinforcing the hypothesis that ORF14 might not be related to xanthan biosynthesis. Further studies are required to determine the function of the ORF14 gene product.

To investigate whether ORF14 belongs to the *gum* operon, subfragments of the *gum* region were cloned into pK18mob or pK19mob cloning vectors. The *gum* genes encoded by the cloned fragments were transcribed in the orientation opposite to that of the *lacZ* α promoter located on the vector. The hybrid plasmids were transferred to XcORF14. Integration of these plasmids, by a single-crossover event, caused interruption of the *gum* region. Transconjugants were verified by Southern hybridization and assayed for their β -galactosidase (β -Gal) activities (Fig. 3B). Integration of pGum01-19AS resulted in strains that displayed β -Gal activities similar to those of the recipient strains without the integrated plasmid (13) (Fig. 3B). Whereas the integration of pGum20-19AS reduced the β -Gal activity mediated by the *gumM* transcriptional fusion to 10% (13) (Fig. 3B), the β -Gal activity of the XcORF14 strain was not significantly affected by the integration of pGum20-19AS or pGum70-18AS. However, the integration of pGum71-18AS reduced the β -Gal activity of XcORF14 more than 50%. These results suggest that transcription of the *gum* operon may terminate within a region of 589 bp between the stop codon of *gumM* and the start codon of ORF14. No clear transcriptional terminators were located in this fragment, although a short open reading frame, not described in the original *gum* sequence, was found. This open reading frame, whose deduced amino acid sequence displayed no homology to those of proteins of known function, was termed ORF13, and it partially overlaps sequences with weak promoter activity located immediately upstream of ORF14, responsible for the reduced β -Gal activity due to integration of pGum71-18AS. These sequences display no clear homology to the proposed *X. campestris* consensus promoter sequences (13) or to *E. coli* σ^{70} promoters.

To determine whether ORF13 and *gumM* belong to the same transcriptional unit, reverse transcriptase (RT) PCR experiments were performed as described previously (23), applying a single amplification round. Whole-cell RNA was isolated from strain FC2 as described previously (13), with a further treatment with RNase-free DNase I (GIBCO BRL, Gaithersburg, Md.). The nucleotide sequences of the primers used were as follows: F1 (5'-CCGACCTGATTCCGTACCTTTG-3'), B1 (5'-GAGAGAAAATCCAGCAAGGCG-3'), F2 (5'-CGCCA GGTGCTGGATGACAG-3'), and B2 (5'-CGAAACCGTGC AGCCAC-3'). A diagram showing the position and orientation of each primer is presented in Fig. 4A. The right primer of each correspondent pair was employed for reverse transcription. The results are shown in Fig. 4B. While internal sequences of *gumM* and ORF13 could be RT-PCR amplified (Fig. 4B, lanes 2 and 5, respectively), the expected 893-bp RT-PCR product encompassing both sequences could not be obtained (Fig. 4B, lane 8). These results indicate that ORF13 is transcribed separately from *gumM*. Therefore, the *gum* transcript terminates within 418 bp downstream of *gumM*. These results, in conjunction with previous data (13), show that the *gum* gene cluster is expressed as a 12-cistron operon, from *gumB* to *gumM*.

Nucleotide sequence accession number. The GenBank accession number of the DNA fragment determined in this work is U70053.

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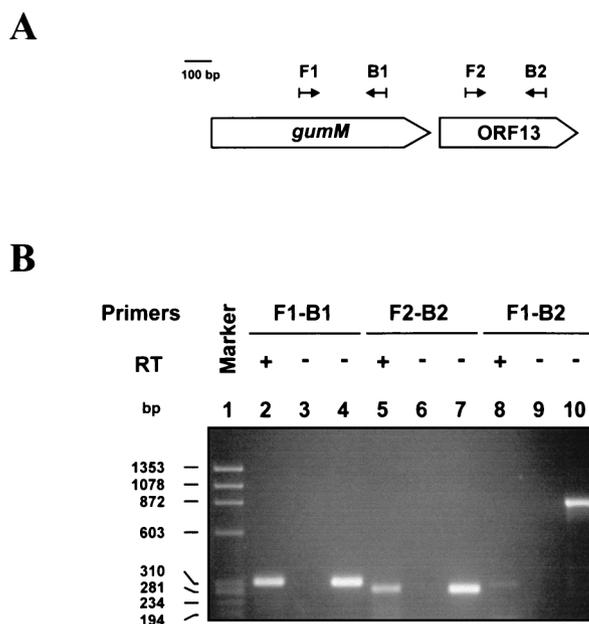


FIG. 4. RT-PCR analysis of the 3' end of the *gum* operon. (A) Scheme showing the position and orientation of the primers (black arrows) in respect to *gumM* and ORF13. (B) Ethidium bromide-stained agarose gel (1.8%) of whole-cell RNA-based RT-PCR (lanes 2, 3, 5, 6, 8, and 9) and genome-based PCR (lanes 4, 7, and 10) products amplified with the following primers: F1 and B1 (lanes 2 to 4), F2 and B2 (lanes 5 to 7), and F1 and B2 (lanes 8 to 10). Controls without RT added were included (lanes 3, 6, and 9). DNA size standards (in base pairs) are indicated (lane 1). +, present; -, absent.

tors, pJC372, and the strain Rm1021, respectively. We thank Pablo Cerdán for assistance in GUS assays and A. J. Parodi for critically reading the manuscript. Photographs were kindly taken by Eduardo J. Rattner.

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