Cell Growth Inhibition upon Deletion of Four Toxin-Antitoxin Loci from the Megaplasmids of *Sinorhizobium meliloti*

Branislava Milunovic, George C. diCenzo, Richard A. Morton, Turlough M. Finan

Center for Environmental Genomics, Department of Biology, McMaster University, Hamilton, Ontario, Canada

Toxin and antitoxin (TA) gene pairs are addiction systems that are present in many microbial genomes. *Sinorhizobium meliloti* is an N\textsubscript{2}-fixing bacterial symbiont of alfalfa and other leguminous plants, and its genome consists of three large replicons, a circular chromosome (3.7 Mb) and the megaplasmids pSymA (1.4 Mb) and pSymB (1.7 Mb). *S. meliloti* carries 211 predicted type II TA genes, each encoding a toxin or an antitoxin. We constructed defined deletion strains that collectively removed the entire pSymA and pSymB megaplasmids except for their oriV regions. Of approximately 100 TA genes on pSymA and pSymB, we identified four whose loss was associated with cell death or stasis unless copies of the genes were supplied in trans. Orthologs of three of these loci have been characterized in other organisms (relBE / sma0471/sma0473), Fic [DOC] / sma2105, and VapC [PIN] / orf2230/sma2231), and this report contains the first experimental proof that RES/Xre (smb21127/smb21128) loci can function as a TA system. Transcriptome sequencing (RNA-seq) analysis did not reveal transcriptional differences between the TA systems to account for why deletion of the four “active” systems resulted in cell toxicity. These data suggest that severe cell growth phenotypes result from the loss of a few TA systems and that loss of most TA systems may result in more subtle phenotypes. These four TA systems do not appear to play a direct role in the *S. meliloti*-alfalfa symbiosis, as strains lacking these TA systems had a symbiotic N\textsubscript{2} fixation phenotype that was indistinguishable from the wild type.

Toxin (T) and antitoxin (A) genes are ubiquitous and somewhat mysterious with respect to function. Type II TA systems are those in which the antitoxin and toxin are both proteins, and these are generally transcribed as antitoxin-toxin gene pairs (1–3). During unperturbed, exponential growth, the toxic activity of the toxin is neutralized through a physical interaction with the antitoxin protein, and in most systems, the TA protein complex also regulates TA transcription by binding to the TA promoter region (3).

Under conditions where the number of toxin molecules is greater than those of the antitoxin, the toxin is free to exert its effect on the cell. Several mechanisms lead to conditions where the toxin molecules outnumber the antitoxin molecules. When a TA pair is lost from the cell, the residual antitoxin degrades faster than the toxin (2, 4). Alternatively, the antitoxins can be degraded by Lon or Clp proteases (2, 5, 6). The loss of the antitoxin releases the toxin, which subsequently interferes with a vital cell process (2, 3, 7). Some toxins insert into and destabilize the membrane, others interact with DNA gyrase and interfere with DNA replication, and others disrupt translation either by functioning as an endoribonuclease or through an interaction with the ribosome (8–10).

Why TA systems are maintained within the genome is an area of active research, and many hypotheses have been put forth (7, 11). The simplest of these models suggest that they are involved in stabilizing plasmids and nonessential chromosomal segments, or possibly that they function as plasmid antiaddiction modules (12, 13). An alternative hypothesis is that they are abortive infection systems involved in a population-level defense against phage infection (14). Other models postulate that TA systems are involved in the metabolic regulation of the cell or help the cell adapt to various stresses via persister cell formation (15–17). Here, we report a deletion analysis of the megaplasmids of the alphaproteobacterium *Sinorhizobium meliloti* and we focus on how the loss of TA genes affects cell growth. *S. meliloti* has a multipartite genome, and it lives both as a free-living soil bacterium and in a symbiosis with *Medicago sativa* and sweet clover (*Melilotus alba*). The model *S. meliloti* strain, 1021, contains a circular chromosome (3.7 Mb) and two megaplasmids, pSymA (1.4 Mb) and pSymB (1.7 Mb) (18–20). Surveys of worldwide isolates show that the presence of two very large megaplasmids and a circular chromosome is a feature of the *S. meliloti* genome. The megaplasmids are notable for their essential role in establishing a successful symbiosis with host plants (19–23). The replicons also appear valuable for free-living growth as they carry loci that increase metabolic diversity, such as a denitrification pathway on pSymA (19, 24, 25), a high density of solute transport systems on pSymB (20, 26, 27), and genes involved in osmoadaptation on pSymB (28). Two essential genes, engA and the only copy of an Arg-tRNA gene, are located on pSymB, and there is strong evidence that the DNA region containing these genes translocated from the chromosome to pSymB in an ancestral strain (29). Megaplasmid pSymA is a nonessential replicon (30). Yurgel et al. (31) recently reported on a collection of 50 SymA deletion derivatives and identified loci involved in cytochrome c activity, growth on various carbon and nitrogen sources, and symbiotic N\textsubscript{2} fixation effectiveness.

Outside the above-mentioned processes, relatively few functions have been attributed to the *S. meliloti* megaplasmids, despite their carrying almost 2,900 predicted protein-coding genes (19, 20). Bioinformatics studies suggest that about 100 megaplasmid...
genes (>3% of the predicted genes) encode toxin or antitoxin proteins (1, 32). Here, we describe the construction of a deletion library that spans both the pSymA and pSymB megaplasmids except for the regions directly surrounding the oriVs. We show that of the ~100 TA genes deleted, loss of only four TA loci was associated with subsequent cell death or stasis. Examination of transcription of the TA genes by transcriptome sequencing (RNA-seq) analysis revealed no correlation between the expression of the TA loci and their growth phenotype. To our knowledge, this work represents the largest systematic deletion analysis of TA genes reported to date.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Table S1 in the supplemental material. All the S. meliloti strains are derived from the top121, derivative, RmP110, which carries a functional pSTC gene (33).

Medium, antibiotics, and growth conditions. Luria-Bertani (LB) medium contained 10 g Difco tryptone, 5 g Difco yeast extract, and 5 g NaCl per liter of H2O. For S. meliloti, LB was supplemented with 2.5 mM MgSO4 and 2.5 mM CaCl2 (LBmc). Minimal medium M9 contained, per liter, 5.8 g Na2HPO4, 3 g KH2PO4, 0.5 g NaCl, 1 g NH4Cl, 1 μg/ml biotin, 10 mg/ml CoCl2, 1 mM MgSO4, 0.25 mM CaCl2, and 10 mM carbon source. For screening compounds as sources of nitrogen, NH4Cl was omitted from M9. Antibiotic (streptomycin [Sm], neomycin [Nm], spectinomycin [Sp], gentamicin [Gm], tetracycline [Tc], and chloramphenicol [Cm]) concentrations in liquid and solid media were as previously described (34, 35). In liquid medium, the antibiotic concentrations were halved. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) were used at the final concentrations of 40 μM and 0.5 mM, respectively. S. meliloti strains were grown at 30°C while Esherichia coli strains were grown at 37°C.

DNA manipulations. Genomic DNA from S. meliloti and plasmid DNA from E. coli were isolated as previously described (36). Agarose gel electrophoresis and other recombinant DNA techniques such as restriction analysis and DNA ligation and transformation were done according to reference 36. DNA sequencing was performed by the MOBX facility at McMaster University, Hamilton, Ontario, Canada.

FRT targeting vector pTH1937. Plasmid pTH1937 carries the p15A arvV from pACYC177 (37); the nptII gene (neomycin resistance) from Tn5; a multiple cloning site (MCS) containing the unique restriction sites Spel, Swal, EcoRV, PvuI, and EcoRI; and the oriT from RK2; and an FLP recombination target (FRT) site. pTH1937 was targeted to specific loci and their growth phenotype. To our knowledge, this work is the first report for the regions directly surrounding the oriV.

Construction of S. meliloti strains carrying two FRT sites. Previously, an S. meliloti RmP110 gene fusion library was constructed through single crossover recombination of pTH1522 into the genome (35). The pTH1522 vector carries an FRT site, and the location of the insertion was previously determined through sequencing. The pTH1522 library strains were used as recipients for a triparental mating with an E. coli strain carrying pTH1937 (FRT, Nm r) with appropriate S. meliloti DNA fragment. Plasmids were mobilized from E. coli to S. meliloti in triparental matings with the E. coli MT616 strain carrying pRK600 (23).

Construction of the flp delivery vector pTH1944. Plasmid pTH1944 was constructed in order to express flp recombinase in S. meliloti. A 2-kb PstI fragment containing the flp gene from pTH472 (40) was cloned into pTH1919 (J. Cheng and T. Finan, unpublished data) to produce pTH1944. In pTH1944, flp is transcribed from the lac promoter (nt 4088 to 4127) and lac operator (lacO) sites are present at nt 4068 to 4091. While pTH1944 does not carry the lacI gene, in some experiments lacI was expressed from the pTH1931 plasmid (29) and flp expression was induced by adding 0.5 mM IPTG to the medium. S. meliloti strain RmH940 contains a lacZ·nptII (Nm r) cassette flanked by FRT sites in direct orientation (40). In control experiments, pTH1944 resulted in 100% excision of the FRT-flanked lacZ·nptII genes in S. meliloti RmH940 when 0.5 mM IPTG was present in the medium. In the absence of IPTG, 90% of the RimH940 transconjugants retained the FRT-flanked lacZ·nptII genes.

Megaplasmid deletions. The majority of the deletions in this study were made via transfer of the FLP recombinase plasmid pTH1944 (Tc r) into the S. meliloti pTH1522/pTH1937 double integrant strains (transconjugation frequency, 10–3 to 10–4/recipient). However, several additional deletions were made by combining two individual FRT-marked deletions by transduction of Gm r Nm r–marked deletions into recipient strain deletion strains that lacked antibiotic resistance markers (see Fig. 1). Prior to these transductions, it was essential to cure the Tc r plasmid from the recipient cells, and this was done by sequential subculturing and screening for Tc r colonies. Strains carrying FRT-flanked regions A150, A152, and A160 were generated as follows: A150 [ΔA127 (Gm r Nm r) → ΔA106] (nt 1898 to 980) (40) and A160 were generated as follows: A160 [ΔA105 (Gm r Nm r) → ΔA102] (nt 10988 to 184519). Subsequent deletion of the regions between the FRT sites was achieved upon conjugation of the flp (pTH1944-Tc r) plasmid into these strains, and the deletions ΔA150, ΔA152, and ΔA160 were generated. ΔB180 was constructed by transducting ΔB143 (nt 635904 to 678812) into ΔB139 (nt 74072 to 869642) without first curing pTH1944.

Cloning toxin-antitoxin gene regions. DNA fragments containing the sma0471/sma0473, sma2151, sma2231/sma2225, sma2273/sma2275, and sma2105 genes; the upstream region of sma2105; the sma2133 and smb2127/smb2112 genes; and their predicted promoter regions were amplified from RmP110 DNA using the primers listed in Table S2 in the supplemental material. The products were cloned as a PacI fragment into the pBluescript II X-Blue vector (16) to produce the pTH2563, pTH2622, pTH2623, pTH2624, pTH2625, pTH2646, pTH2790, pTH2647, and pTH2830, respectively.

Growth curves and viable cell counts. Cultures were grown and aerated on a roller drum (Rollordrum; New Brunswick Scientific Co., Edison, NJ, USA) at 30°C. Strains grown overnight in LBmc medium were subcultured to an optical density at 600 nm (OD600) of 0.03 to 0.04 in 5 ml LB medium with or without IPTG (0.5 mM). Growth was measured by monitoring the OD600 of the culture every 3 h. The number of CFU was determined by plating samples on LB medium with or without IPTG (cultures without IPTG were plated on plates without IPTG and cultures with IPTG were plated on plates with IPTG, except for the zero time point).

812 jb.asm.org
Transcription analysis. Makarova et al. (1) identified 211 putative toxin or antitoxin genes in the *S. meliloti* 1021 genome. Forty-two of these genes occur within 21 contiguous gene pairs. Expression of putative toxin-antitoxin genes was examined using RNA-seq data. Briefly, RNA was prepared from *S. meliloti* P110 grown in morpholinepropanesulfonic acid (MOPS) minimal medium containing 20 μM (MOPS-P0) or 2 mM (MOPS-P2) inorganic phosphate, respectively. A directional cDNA library was prepared, and Illumina sequence reads of 34 nucleotides were obtained from it by Fasteris SA (Geneva, Switzerland). Sequence reads were aligned with the *S. meliloti* 1021 genome, and data were deposited in the GEO public functional genomics data repository under accession number GSE43558. In addition, we obtained public RNA-seq data for *S. meliloti* strain 2011 from GEO sequence accession no. GSE44083 as described by Sallet et al. (41). These data were combined, and a summary of the RNA expression of the 211 toxin-antitoxin genes that includes the *S. meliloti* gene names, the TA protein class, GI numbers, and location of each in the *S. meliloti* genome is given in Table S3 in the supplemental material. The data counts are expressed as the number of times that the 5′ end of an RNA-seq read mapped to a nucleotide within the gene regions.

Nucleotide sequence accession number. The 2,638-nucleotide sequence for pTH1937 has been deposited in GenBank under the accession number KF188463.

Microarray data accession number. Data for *S. meliloti* RmP110 were deposited in the GEO public functional genomics data repository under accession number GSE43558.

RESULTS

FRT-directed deletion strategy using targeting vectors pTH1522 and pTH1937. To delete DNA regions via Flp/FRT-directed recombination, FRT sites were inserted in direct orientation on each side of the genome region to be excised (schematic in Fig. 1). One FRT site was from a reporter fusion library of 6,000 strains in which the Gm' suicide plasmid pTH1522, carrying a single FRT site, is integrated via single crossover recombination at known locations throughout the *S. meliloti* genome (35). The second FRT site was from the Nm' suicide plasmid pTH1937, which carries an FRT site and a pSC101 oriV (see Materials and Methods). Plasmids pTH1937 and pTH1522 share insufficient homology to direct recombination, and in control experiments, no Nm' transconjugants (<10−8/recipient) were detected when pTH1937 was transferred into an *S. meliloti* strain carrying an integrated pTH1522. To direct pTH1937 to a particular location in the *S. meliloti* genome, 0.5 to 1.5 kb from the target region was cloned into pTH1937 and the resulting pTH1937 derivative was transferred into the target region of the appropriate *S. meliloti* pTH1522 integrant strain (Fig. 1).

To catalyze recombination between the FRT sites, the tetracycline-resistant (Tc') Flp recombinase plasmid pTH1944 was

---

**Fig 1** Schematic showing the strategy employed to generate FRT-directed deletions. Two different deletion phenotypes/genotypes result when pTH1937 integrates on either side of a single pTH1522 integrant in the genome. BC, FG, and KL are random DNA sequences that were cloned into targeting vectors. Gm', gentamicin resistance; Nm', neomycin resistance; FRT, Flp recombinase target site; reporter genes, gusA and lacZ; GFP, green fluorescent protein; RFP, red fluorescent protein; Δ-DE, deleted region 1; Δ-HJ, deleted region 2.
transferred into the *S. meliloti* ΦpTH1522ΦpTH1937 double integrant strains (10^−4/recipient). Depending on whether the integrated pTH1522 lay upstream or downstream from the integrated pTH1937, the resulting deletion strain was either Sm rif Gm rif Nm rif Tcr, with the pTH1522-carried *lacZ* gene lost, or Sm rif Gms Nms Tcr, with the pTH1522-carried *lacZ* gene retained (Fig. 1, DE and HJ, respectively).

We note that even when the deleted region carried essential genes, Tcr Flp recombinant transconjugants were still recovered, albeit at a reduced frequency. These transconjugants arose as a result of loss of one of the FRT sites by spontaneous RecA-directed resolution of the ΦpTH1522 cointegrate or of the ΦpTH1937 cointegrate. Hence, Tc Flp recombinase plasmid transconjugants were screened for their Gm', Nm', and LacZ phenotypes (Fig. 1), and as described below, purified putative deletion strains were then analyzed by PCR. **Construction and confirmation of the pSymA and pSymB megaplasmid deletions.** Eighty-nine FRT-flanked regions of pSymA or pSymB were assigned ascending numbers from A100 and B100, respectively. The precise boundaries of the FRT sites are given in Table S1 in the supplemental material, and schematics showing a subset of the regions from pSymA and pSymB are shown in Fig. 2 and 3, respectively. Following transfer of the Flp recombinase to these strains, PCR was used to amplify and detect gene regions located inside and outside the FRT deletion end-points and thus confirm, or not, that the FRT-flanked regions were deleted. For example, Fig. 4 shows a schematic and the PCR products obtained for the B123 region of pSymB before and after transfer of the Flp plasmid to the B123 strain. PCR products internal to the B123 region are clearly present in the B123 strain prior to transfer of the Flp plasmid (Fig. 4, lanes 6 and 7), while these fragments are absent in the B123 strain carrying the Flp
plasmid (lanes 2 and 3, loci C and D). A region from the chromosome was amplified as a control in all reactions (region Y in Fig. 4). These data confirmed the gross structure of the \( \Delta H9004 \) B123 deletion, and all of the deletion strains were similarly confirmed by PCR (see Table S1). When the deletion was verified with respect to the presence/absence of antibiotic resistance and PCR markers, the deletion was designated with a \( \Delta H9004 \) (e.g., \( \Delta H9004 \) B118).

**Megaplasmid pSymA and pSymB deletion analyses.** Individual deletion strains (\( \Delta A102 \) to \( \Delta A133 \)) which together removed all of pSymA except the 10-kb \( \text{oriV} \) region were recovered (e.g., \( \Delta A101 \)). The isolation of the \( \Delta A102 \) to \( \Delta A133 \) strains suggested that pSymA does not carry any essential genes, as expected considering that pSymA has previously been cured from \( S. \) meliloti (22). We therefore expected that the transfer of Flp recombinase to pSymA carrying the FRT-flanked A101, A134, or A135 region would result in the recovery of strains that retained the A101, A134, or A135 region and lost the remainder of pSymA; however, no such strains were recovered. These results, together with findings that several of the deletions that were generated via Flp recombinase could not be transferred (transduced) into the wildtype RmP110 (see below), led us to identify three pSymA regions that carried active toxin-antitoxin systems.

The pSymB megaplasmid is 1,683,333 bp in size, and its DNA sequence is numbered clockwise, with nucleotide position 1 annotated as the A of the \( \text{lacE} \) gene (4). Fifty-six double integrant strains that carried FRT sites in direct orientation in the pSymB megaplasmid were made (Fig. 3). As outlined previously, deletions that removed the B110 and B117 regions were recovered only when the essential \( \text{engA} \) (B110) and Arg-tRNA (B117) genes were present in trans on a replicating plasmid or integrated into the chromosome (29). Deletion mutants that removed the \( \text{smb20056-smb20057-smb20058} \) genes encode an ABC-type cobalt transport system (\( \text{cbtJKL} \)) required for growth of RmP110 in LB (42). Furthermore, the inability to efficiently transfer the megaplasmid deletions into wild-type recipient strains by...
transduction led to the identification of an active pSymB toxin-antitoxin system (see below).

However, even when engA and the Arg-tRNA were present in trans, and excess cobalt (2 ng/ml) was included in the medium, deletions that remove the pSymB oriV region (e.g., ΔB125) were not recovered. Thus, for both pSymA and pSymB, deletions that removed the megaplasmid replication origins were not recovered.

Identification of toxin-antitoxin systems. In performing a genetic analysis of the pSymA and pSymB deletion strains, we observed that the Gmr Nmr-marked deletions ΔA131 (nt 1173730 to 1231998), ΔA132 (nt 1232916 to 1283082), ΔA257 (nt 255655 to 262769), and ΔB139 (nt 740722 to 869642) could not be recombined via transduction into the wild-type recipient RmP110. In control experiments, the individual FRT insertions used in the construction of the ΔA131, ΔA132, ΔA257, and ΔB139 deletions were readily recombined via transduction into wild-type strain RmP110 (frequency, ~10^-7/recipient). These data suggested that loss of a gene(s) within the A131, A132, A257, and B139 regions prevented recovery of ΔA131, ΔA132, ΔA257, and ΔB139 recombinants, respectively, and below, we show that the responsible genes encoded toxin-antitoxin systems. We note that while the ΔA131, ΔA132, ΔA257, and ΔB139 deletion strains were originally recovered via Flp recombinase-directed deletion, in repeat experiments the recovery of these deletions was observed to occur at a very low frequency.

Bioinformatic analysis of the S. meliloti 1021 genome identified 211 genes predicted to encode type II toxin or antitoxin proteins (1, 32). We extracted these genes from supplemental data reported by Makarova et al. (1), and a summary showing the distribution of these TA genes among the three S. meliloti replicons is shown in Table 1. The locations of the TA-associated genes on the pSymA and pSymB megaplasmids are shown in Fig. 2 and 3, and detailed coordinates for each gene are given in Table S3 in the supplemental material.

Toxin-antitoxin gene sma2105 on the pSymA megaplasmid. The inability to transduce ΔA131 (nt 1173730 to 1231998) into RmP110 prompted an examination of the genes present in the 60-kb A131 region (Fig. 5B). Three genes, sma2105 (1,221 bp), sma2133 (792 bp), and sma2151 (324 bp), are predicted members of the toxin-antitoxin families Fic, Xre, and Xre, respectively (1, 19). These loci, together with the upstream promoter regions, were cloned into the Sp' pTH1931 vector to generate the plasmids pTH2646, pTH2647, and pTH2622, respectively. When RmP110 carried the sma2105 gene (pTH2646) in trans, transduction of the Gmr Nmr-marked ΔA131 deletion into wild-type RmP110 occurred (frequency, ~10^-7/recipient), while no transductants were obtained when sma2133 (pTH2647) or sma2151 (pTH2622) was present in trans (Table 2 and data not shown). The pTH1522 integrant (FL4094) used in the construction of ΔA131 was readily transduced into all these strains. The role of the region upstream of the sma2105 gene in transduction of the Gmr Nmr-marked ΔA131 deletion was also examined. The region upstream of sma2105 (nt 1187240 to 1187703) was cloned in the pTH1931 vector as a 463-bp fragment to generate the plasmid pTH2790 (Fig. 5B). Transduction of the Gmr Nmr-marked ΔA131 deletion into wild-type RmP110 occurred (frequency, ~10^-7/recipient) when RmP110 carried the upstream region of sma2105. The region upstream of sma2105 therefore appears to carry an antitoxin gene, and how this controls toxin activity requires further investigation.
Toxin-antitoxin system orf2230/sma2231 on the pSymA megaplasmid. The 53-kb ΔA132 deletion (nt 1232916 to 1283082) could not be transduced into the wild-type RmP110, and three toxin-antitoxin loci lay within this region: sma2231 (384 bp), sma2253/sma2255 (378/339 bp, respectively), and sma2273/sma2275 (351/276 bp, respectively) (1). While sma2273 initially appeared to be an isolated toxin gene, analysis of the 754-nt region upstream of sma2231 allowed us to isolate a putative open reading frame (Fig. 5C). This open reading frame, designated orf2230, encoded a 66-amino-acid protein whose TTG stop codon overlaps the sma2231 GTG start codon by a single nucleotide. We sought to clone the orf2230/sma2231, sma2253/sma2255, and sma2273/sma2275 genes with their corresponding promoter regions into the Sp′ pTH1931 vector. The clone of sma2253/sma2255 together with a possible 66-amino-acid antitoxin upstream of sma2253 was not recovered in E. coli; however, pTH1931 clones carrying only sma2253 and the putative upstream antitoxin were recovered. orf2230/sma2231 and sma2273/sma2275, together with their promoter regions, were cloned into pTH1931 to form pTH2623 and pTH2625, respectively. Transduction of ΔA132 (Gm′ Nm′) into RmP110 carrying these plasmids occurred only when RmP110 carried the orf2230/sma2231 genes in trans (frequency, 1 × 10⁻⁷ recipient) (Table 2). No recombinants were obtained when ΔA132 was transduced into RmP110 carrying sma2253 or sma2275/sma2275 in trans.

Toxin-antitoxin system sma0471/sma0473 on the pSymA megaplasmid. The ability to transduce ΔA152 (nt 402136 to 1122176) and not ΔA150 (nt 186200 to 930000) into RmP110 suggested that a gene(s) within the nt 193611 to 400267 region was responsible for this phenotype. Further deletion analysis of this large region (data not shown) allowed us to localize the region that was recalcitrant to deletion to a 9-kb region (nt 255655 to 264969) carrying nine annotated genes. Of these genes, sma0471/sma0473 were identified as responsible for the failure to transduce ΔA257 (nt 255655 to 262769), as the presence of these genes in trans on a replicating plasmid (pTH2563) (Fig. 5A) in the RmP110 recipient allowed transduction of the ΔA257 deletion (10⁻⁷ recipient) (Table 2).

Toxin-antitoxin system smb21127/smb21128 on the pSymB megaplasmid. Very few colonies were recovered upon transduction of the ΔB139 (nt 740722 to 869642) deletion into RmP110 (10⁻⁷ recipient). Examination of this region revealed a putative TA locus, smb21127/smb21128, and, at a separate location, a single putative antitoxin gene, smb21117. As the smb21127/smb21128 locus is the only one predicted to encode a toxin, this gene pair was cloned into pTH1931, producing pTH2830 (Fig. 5D). The presence of these genes in trans, or just the putative antitoxin smb21127, allowed the recovery of ΔB139 transductants at a frequency of 10⁻⁷ recipient (data not shown). Furthermore, disruption of the putative toxin gene smb21128 allowed subsequent transduction of ΔB180 (nt 635940 to 869642) at a frequency of 10⁻⁷ recipient (data not shown). These data confirmed that smb21127/smb21128 encode an active toxin-antitoxin system.

Effect of TA systems on the growth of S. meliloti. The effect of the toxin-antitoxin-like loci on the growth and cell viability of S. meliloti was monitored by deleting the megaplasmid regions in strains with or without the TA loci in trans. In the trans copy of the appropriate TA loci was present as an insert on the replicating pTH1931 (Sp′) plasmid, and the FRT-flanked regions were then deleted via the IPTG-dependent induction of flp from pTH1944 (Tc′). Additional control strains included a strain in which FRT sites flanked the pSymA oriV (A101), a strain in which FRT sites flank a pSymA region that could be deleted without any apparent effect (A128), and a strain in which the essential Arg-tRNA gene of pSymB is flanked by FRT sites (B158). All these strains were grown overnight in LBmc medium and diluted to an OD₆₀₀ of 0.03 to 0.04, and half of the resulting cultures were grown with 0.5 mM IPTG and half without IPTG. The optical density (OD₆₀₀) and cell viability of the resulting cultures were monitored over a 24-h period (Fig. 6).

In all experiments, deletion of the tRNAArg gene region (B158) and the pSymA oriV (A101) region resulted in decreased growth and 100-fold and 1,000-fold drops in cell viability, respectively (Fig. 6). Deletion of the sma2105 and orf2230/sma2231 TA loci also appeared to have a bactericidal effect, as a >10-fold drop in cell viability was observed, while the deletion of sma0471/sma0473 and smb21127/smb21128 loci was bacteriostatic. Importantly, the presence of the appropriate TA loci in trans appeared to completely rescue the observed growth phenotypes, while none of the TA loci individually rescued the decrease in cell density and viability that resulted from the deletion of the pSymA oriV (ΔA101).

**TABLE 1** Predicted toxin and antitoxin protein classes in the genome of S. meliloti 1021

<table>
<thead>
<tr>
<th>Type</th>
<th>No. found in:</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSymA</td>
<td>pSymB</td>
</tr>
<tr>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>AbdB</td>
<td>2</td>
</tr>
<tr>
<td>ArsR</td>
<td>2</td>
</tr>
<tr>
<td>COG2442</td>
<td>1</td>
</tr>
<tr>
<td>COG2856</td>
<td>1</td>
</tr>
<tr>
<td>COG2929</td>
<td>1</td>
</tr>
<tr>
<td>COG3832</td>
<td>3</td>
</tr>
<tr>
<td>COG5642</td>
<td>2</td>
</tr>
<tr>
<td>COG5654</td>
<td>2</td>
</tr>
<tr>
<td>Fic</td>
<td>2</td>
</tr>
<tr>
<td>GNAT</td>
<td>2</td>
</tr>
<tr>
<td>HEPN</td>
<td>1</td>
</tr>
<tr>
<td>HicA</td>
<td>1</td>
</tr>
<tr>
<td>HicB</td>
<td>3</td>
</tr>
<tr>
<td>HipA</td>
<td>1</td>
</tr>
<tr>
<td>MazF</td>
<td>2</td>
</tr>
<tr>
<td>MazFn</td>
<td>1</td>
</tr>
<tr>
<td>MNT</td>
<td>1</td>
</tr>
<tr>
<td>PHD</td>
<td>1</td>
</tr>
<tr>
<td>PIN</td>
<td>8</td>
</tr>
<tr>
<td>RelE</td>
<td>5</td>
</tr>
<tr>
<td>RHH</td>
<td>5</td>
</tr>
<tr>
<td>Xre</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
</tr>
</tbody>
</table>

a Data were extracted from TA genes predicted by Makarova et al. (1).

b Sma2105 has two Fic domains, Smb20754 and Smbc0769 each have COG2856 and Xre domains, and Smb20835 has HEPN and MNT domains. The open reading frame upstream of sma2231 is not included in the table.
production of the toxin and that of the antitoxin are simultaneously disrupted, the residual antitoxin quickly degrades, liberating the toxin to exert its effect. Thus, when a TA locus is lost from a cell, potentially through a genome deletion event, the toxin is released and is generally expected to promote cell death or stasis in a large majority of the population. We identified only four TA loci whose deletion dramatically affected cell growth (sma0471/sma0473, sma2105, orf2230/sma2231, and smb21127/smb21128), and we discuss these loci below.

The 102- and 97-amino-acid proteins encoded by sma0471 and...
sma2105 locus resulted in a bactericidal effect [Fig. 6, A131(pTH1931) versus A131(pTH1931) (+ IPTG)].

Examination of both RNA-seq data and alignments of Sma2105 and its orthologs indicated that the actual ATG start codon for Sma2105 is 14 amino acids further into the gene than originally annotated in Rm1021, and this is corrected in the Rm2011 annotation (18, 41). Interestingly, sma2105 is annotated as being carried by a monocistronic mRNA, without a cognate antitoxin. Moreover, there is a large 500-nt region upstream of sma2105 with no annotated gene, and plasmid clones carrying only this region allowed the deletion of sma2105 (Table 2). There is no evidence of a protein-coding region or small RNA present in this upstream region; therefore, it seems unlikely that this upstream region encodes an antitoxin to the Sma2105 toxin, although this cannot be fully ruled out. An intriguing possibility is that Sma2105 represents a novel toxin-antitoxin family. Unlike other Doc proteins, Sma2105 appears to contain a MarR-like DNA-binding domain in the C-terminal domain. Perhaps, Sma2105 binds to the region upstream of sma2105, and this association prevents the protein from exerting its toxic effect; thus, only when the concentration of Sma2105 exceeds that of its DNA-binding site would it show a bactericidal effect. Further studies are required to resolve the organization of this locus and the nature of this predicted interaction.

The third toxic TA system encodes the Orf2230 antitoxin and the Sma2231 toxin proteins. Sma2231 is a member of the ∼130-amino-acid PIN-domain protein family, whose proteins are often the toxic components of vapBC TA systems (50). VapC toxins containing a PIN domain block protein translation via mRNA cleavage (51). In vapBC systems, the VapB protein is generally coexpressed with VapC and it inhibits VapC RNase activity. Makarova et al. (1) reported that 21 of 24 PIN-domain proteins in S. meliloti are present as vapBC TA systems (12 on chromosome, 8 on pSymA, and 4 on pSymB). While sma2231 initially appeared to be an isolated vapC-like gene, there is a 754-nt region upstream of sma2231. Analysis of this region allowed us to identify a putative open reading frame, designated orf2230, upstream of sma2231 encoding a 66-amino-acid protein. As is common in vapBC operons, the TTG stop codon of orf2230 overlaps the sma2231 GTG start codon by a single nucleotide (50). The Orf2230 protein contains a domain of unknown function (DUF2191), which is also present in multiple VapB homologs in Mycobacterium tuberculosis (51). Unlike the bacteriostatic effect observed to be associated with VapC toxins from Mycobacterium smegmatis and M. tuberculosis, our analysis showed that deleting orf2230/sma2231 (∆A132) had a bactericidal effect (52, 53).

The only active TA system detected on pSymB was the smb21127/smb21128 locus. The gene for the predicted toxin, Smb21127, encodes a 184-amino-acid hypothetical protein from the COG5649 and pflm09722 families. These proteins share a RES domain, so named because they share three highly conserved polar amino acids, arginine (R), glutamate (E), and serine (S), which possibly form an active site with nuclease activity (54). Smb21127 encodes a 123-amino-acid hypothetical antitoxin from the COG5642 and pflm09722 families, and these proteins carry an Xre DNA-binding domain that is commonly found in phage repressor proteins (55). Based on a bioinformatics analysis, Makarova et al. (1) suggested that COG5654-Xre (COG5642) gene pairs could function as TA systems. However, we are unaware of any experimental data to confirm this hypothesis. Our data

---

TABLE 2 Frequency of recombination of S. meliloti megaplasmid deletions into wild-type strains carrying the empty plasmid vector pTH1931 or pTH1931 containing TA genes

<table>
<thead>
<tr>
<th>Phage donor</th>
<th>recipient strain</th>
<th>Transductant colonies per plate (frequency/recipient)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΦΔA257</td>
<td>WT(pTH1931-sma0471/73)</td>
<td>120 (1 × 10^7)</td>
</tr>
<tr>
<td>ΦΔA257</td>
<td>WT(pTH1931)</td>
<td>2 (2 × 10^7)</td>
</tr>
<tr>
<td>ΦΔA131</td>
<td>WT(pTH1931-sma2105)</td>
<td>99 (4 × 10^7)</td>
</tr>
<tr>
<td>ΦΔA131</td>
<td>WT(pTH1931-upstream region of sma2105)</td>
<td>20 (1 × 10^7)</td>
</tr>
<tr>
<td>ΦΔA131</td>
<td>WT(pTH1931)</td>
<td>0 (&lt;10^-6)</td>
</tr>
<tr>
<td>ΦΔA132</td>
<td>WT(pTH1931-sma2230/31)</td>
<td>30 (1 × 10^7)</td>
</tr>
<tr>
<td>ΦΔA132</td>
<td>WT(pTH1931)</td>
<td>0 (&lt;10^-6)</td>
</tr>
<tr>
<td>ΦΔB139</td>
<td>WT(pTH1931-smb21127/28)</td>
<td>107 (4 × 10^7)</td>
</tr>
<tr>
<td>ΦΔB139</td>
<td>WT(pTH1931)</td>
<td>5 (1 × 10^{-5})</td>
</tr>
</tbody>
</table>

*The diagram above the table illustrates the two homologous recombination events (dashed lines) that occur in transduction of deletion ΔCDF::Gm' Nm' from a donor strain into a wild-type recipient. The structure of the resulting Gm' Nm' transductant (recombinant) is shown. Similarly, the Gm' Nm'-marked ΔA257, ΔA131, ΔA132, and ΔB139 deletions (Fig. 2 and 3) were transduced using phage ΦM12 into RmP110 wild-type (WT) recipient strains carrying the empty pSp vector pTH1931 or pTH1931 carrying the TA gene regions as indicated.

*Numbers of transductant colonies per plate as well as transductional frequency per recipient for each transduction are indicated. Results are representative of three independent experiments.

---

sma0473 are closely related to the RelB antitoxin and RelE toxin proteins, respectively (43). The RelBE family is distributed in a wide variety of bacteria and archaea (1, 43). RelE toxicity results from cleavage of mRNA at the ribosomal A site as it is being translated (44). In a recent report, six RelE-like proteins from different phyla, including the 98-amino-acid SM00822 protein from the chromosome of S. meliloti, were shown to inhibit translation by cleaving ribosome-associated transcripts (45). In general, cells exposed to the RelE toxin show a drastic growth arrest (39, 46, 47). In accordance, the deletion of sma0471/sma0473 in S. meliloti resulted in a bacteriostatic phenotype [Fig. 6, A257(pTH1931) versus A257(pTH1931) (+ IPTG)].
FIG 6 Effects on the growth of *Sinorhizobium meliloti* resulting from the Flp-catalyzed deletion of four separate TA loci. Cells were grown in LBmc, and Flp recombinase was induced by addition of IPTG at time zero. Growth was monitored by measuring the optical density at 600 nm, and viable cell count was measured as CFU. The TA loci were deleted in strains carrying the cognate TA gene region in *trans* on the plasmid pTH1931 and in strains carrying empty pTH1931. In addition, all experiments included the three strains A101, A128, and B158. In *S. meliloti* strain A101, the oriV of pSymA is flanked by FRT sites. In strain A128, FRT sites flank a nonessential region of pSymA that carries six TA-associated genes. Strain B158 carries FRT sites flanking the essential tRNA^Arg^ gene.
showed that the deletion of \textit{smb21127/smb21128} had a bacteriostatic effect on \textit{S. meliloti} (Fig. 6), and in further work, we found that providing the antitoxin \textit{smb21127} on pTH1931 in \textit{trans} was sufficient to allow deletion of the \textit{smb21127/smb21128} region. These data suggest that the \textit{smb21127/smb21128} operon functions as an active toxin-antitoxin system and provide the first experimental evidence that COG5654-COG5643 gene pairs can function as TA systems.

Of the ~100 TA-associated genes that have been identified on the pSymA and pSymB megaplasmids (Table 1), this deletion analysis identified only four “active” systems. In comparison, 30 of 88 putative TA systems of \textit{M. tuberculosis} were shown to be functional (51). We were curious as to why so few of the predicted TA systems showed a severe growth phenotype following their deletion. One possibility is that many of the predicted TA loci do not function as TA systems. These loci were predicted to function as TA systems based on a bioinformatics approach; thus, it is possible that some were misclassified (1). Alternatively, some of these loci may contain TA system pseudogenes whose activity has decayed. For example, studies of the \textit{cdq150} toxin in \textit{E. coli} indicate that there are several alleles of this toxin, 30% of which are not toxic (56).

While it is likely that some of the megaplasmid systems are indeed not active, we suspect that many are functional. In TA systems, the toxin-antitoxin protein complex generally negatively regulates its own expression; it is only following protease (Lon or Clp)-mediated degradation of the antitoxin that the locus is expressed at high levels (2, 6). We hypothesized that many of the TA systems may be expressed at low levels, and if functional, there may be insufficient toxin present to kill the cell following deletion of the locus. We therefore investigated the expression of the TA genes of \textit{S. meliloti} by analyzing strand-specific RNA-seq data obtained with RNA from wild-type (RmP110) cells grown with excess phosphate and under phosphate-limiting stress conditions. We also examined RNA-seq data from a recently published \textit{S. meliloti} 2011 (which is derived from the parent strain SU47, as is RmP110) data set obtained from exponentially growing and stationary-phase cells, as well as from bacteroids isolated from 10-day-old nodules from \textit{Medicago truncatula} (41). Interestingly, while \textit{sma0471/sma0473} and \textit{smb21127/smb21128} were highly expressed relative to the other TA genes on pSymA and pSymB, the \textit{sma2105} and \textit{sma2231} genes were expressed at quite low levels. Moreover, the patterns of expression of the megaplasmid genes and those on the chromosome were similar (Fig. 7A and B; see also Table S3 in the supplemental material). Hence, the toxicity of the “active” TA genes does not appear to be a direct reflection of their level of transcription prior to deletion.

The ability to identify active toxin-antitoxin modules through a deletion analysis may also be affected by cross-interactions between the various systems. While some studies in \textit{Caulobacter crescentus} and \textit{M. tuberculosis} suggest that TA pairs belonging to the same family are independent functional units, other studies with \textit{M. tuberculosis} and \textit{E. coli} suggest that TA systems interact with each other both transcriptionally and via modulation of toxin activities (51, 57–60). Of 211 TA-associated genes in \textit{S. meliloti}, representatives include 54 Xre proteins (27 chromosomal), 24 PIN proteins (12 chromosomal), 22 COG3832 orthologs (14 chromosomal), and 16 RelE orthologs (8 chromosomal) (Table 1). Thus, many putative interactions may exist that could mask the activity of these loci when deleted individually; clearly unraveling such interactions will require further studies.

Although not always the case, various studies have shown a link between TA modules and many biological functions (7, 11). Toxin-antitoxin loci were initially identified due to their ability to stabilize plasmids through mediating postsegregation killing (12). Subsequently, chromosomal TA systems were suggested to stabilize large, nonessential chromosomal regions. The “active” TA genes in the \textit{S. meliloti} megaplasmids may prevent the deletion of large regions from these megaplasmids and contribute to the overall stability of these replicons. While pSymA has previously been removed from \textit{S. meliloti} in its entirety through sequential deletions (30), we were unable to remove pSymA in a single step. Experiments are under way to simultaneously provide the antitoxins for \textit{sma0473}, \textit{sma2105}, and \textit{sma2231} in trans to determine if this will allow for all of pSymA to be simultaneously deleted. In addition to stabilization, TA systems have been suggested to function as a defense mechanism against phage infection, in adaptation to stress, and as a regulator of developmental processes, including pathogenesis, biofilm formation, and sporulation (7, 11).

Although we have not analyzed the TA systems reported here in respect to their biological role, an intriguing possibility is that they could be involved in the developmental process leading to a successful symbiosis with host legumes. Indeed, deletion of the \textit{vapBC}-type TA module, designated \textit{bat/bto}, from the soybean symbiont \textit{Bradyrhizobium japonicum} resulted in a reduced symbiotic phenotype, although this may have been due to an indirect pleiotropic effect as the deletion mutant had altered cell morphol-
ogy and metabolism and grew slowly in rich medium (61). Dusha and coworkers also characterized a vapBC-like locus, ntrPR, in S. meliloti, which appears to be involved in the regulation of symbiotic nitrogen fixation. Deletion of the locus improves the symbiotic phenotype of S. meliloti (62–64). Mutations within ntrR also resulted in a pleiotropic effect, with a total of 440 transcripts showing differential abundance compared to the wild type under either aerobic or microaerobic conditions (63). In wild-type bacteria, large-scale transcriptomic changes occur during symbiosis, and the potential for these to be regulated through the activity of TA systems is intriguing (64). Other studies have suggested that TA systems are involved in the selective expression of a subset of genes during developmental processes such as biofilm formation, sporulation, and pathogenesis (65–67). Dry weight measurements of alfalfa plants inoculated with the S. meliloti deletion strains that lack each of the four TA loci identified here were indistinguishable from those of plants inoculated with the wild type (data not shown). Hence, these TA systems did not appear to have any effect on symbiotic N₂ fixation with alfalfa. However, further studies are necessary to investigate whether the TA systems might play a role in the bacteroid death process that accompanies nodule senescence.

Here, we have focused on the use of the megaplasmid deletion strains in the identification of active toxin-antitoxin loci; however, this strain collection is a valuable tool for studies looking to unravel how these megaplasmids contribute to the biology of the cell. This strain collection has previously been employed in the identification of essential genes, a cobalt transport system, and a hydroxyproline transport and metabolic gene cluster (29, 42, 68). The availability of this strain collection will aid researchers in future studies examining the contributions of pSymA and pSymB to both free-living and symbiotic S. meliloti cells.
ACKNOWLEDGMENTS
This work was supported by grants to T.M.F. from the Natural Sciences and Engineering Research Council of Canada. T.M.F. also acknowledges support from Genome Canada, OGI, and the Ontario Research and Development Challenge Fund.

We are very grateful to Marie Elliot and Ye Zhang for insights, interest, and advice and Erika Sallet for clarifications regarding the Rm2011 RNA-seq data.

REFERENCES


