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Regulatable Vectors for Environmental Gene Expression in *Alphaproteobacteria*

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Two expression vectors utilizing the inducible taurine promoter (*tauAp*) were developed. Plasmid pLMB51 is a stable low-copy vector enabling expression in the environment and *in planta*. The higher copy number pLMB509 enables BD restriction-independent cloning, expression, and purification of polyhistidine-tagged proteins.

The *Alphaproteobacteria* are an ecologically diverse class of bacteria that are a major constituent of both aquatic and terrestrial environments. Examples of this group include the extensively studied and agronomically important nitrogen-fixing rhizobia and the metabolically diverse *Rhodobacter*. For such an important group of bacteria, there is a need to develop tools to facilitate genetic analysis in the laboratory and in the environment.

The segregational stability of inducible vectors is particularly important when considering them for use in bacteria growing in the environment, where continuous selection is difficult to maintain. The copy number of the vector should also be low in order to prevent overexpression. Conversely, a higher copy number vector is more suitable for gene expression in the laboratory. In both instances, an important consideration when developing vectors is the selection of a highly inducible promoter to drive gene expression that has a low-uninduced background signal. The *lacZ* promoter from *Escherichia coli* is widely used to regulate gene expression and, along with its repressor *lacI*, has been used successfully in *Alphaproteobacteria* (6, 8). However, in a previous study in which the solute binding protein (SBP)-dependent transporters in the alphaproteobacterium *Sinorhizobium meliloti* strain 1021 (10) were characterized, we identified a tightly regulated and highly inducible promoter *tauAp* (SMb21526). This promoter is from an ATP binding cassette (ABC) transporter that is induced by the amino acid derivative taurine.

We developed two regulatable vectors for use in *Alphaproteobacteria* utilizing *tauAp* (Fig. 1A). The first vector (pLMB51) is based on the highly stable vector pJP2 (incP incompatibility group) (13) and has a copy number of 5 to 7 (4). The second vector (pLMB509) is based on pRU1097 (pBBR incompatibility group) (7) and has a copy number of around 10 (3). These vectors are available through Addgene (http://www.addgene.org/). The construction of the vectors is described in the supplemental material.

Regulation of the *tauA* promoter. Two plasmids, one with *tauAp* cloned upstream of a *gfpmut3.1* reporter gene (pRU1357) (10) and the promoterless parent vector (pRU1097) (7), were conjugated (12) into *Rhizobium leguminosarum* strain Rlv3841 and *S. meliloti* 1021. When induced with 1 mM or 10 mM taurine added to tryptone-yeast extract (TY) agar (2) containing 20 μg ml^{-1} gentamicin (Fig. 2), expression was only observed in the *S. meliloti* background. This suggests positive regulation of *tauAp* by the endogenous TauR regulator, which is present in *S. meliloti* 1021 but not *R. leguminosarum* Rlv3841. Therefore, in the subsequent construction of vectors, *tauR* was cloned along with *tauAp* to enable expression in a wider range of nonnative alphaproteobacterial hosts.

Highly inducible and stable expression vector pLMB51. Plasmid pJP2 is a stable low-copy *gusA* reporter probe vector that contains an active partitioning system (*parABCDE* genes), preventing segregational instability. The vector has been shown by histological staining to be environmentally stable, being retained in bacteroids in legume nodules over several weeks (13). To construct pLMB51, *tauAp* and *tauR* were cloned upstream of the *gus*A reporter in pJP2.

To test the inducibility of the expression vector, pLMB51 was conjugated into *S. meliloti* 1021 and *R. leguminosarum* Rlv3841 and grown with differing taurine concentrations in either general minimal salts (GMS) (10) or acid minimal salts (AMS) (11), respectively, supplemented with 2 μg ml^{-1} tetracycline, 10 mM D-glucose, and 10 mM ammonium chloride. The resulting GusA activity was measured (Fig. 3) as described previously (7). As expected, with a low concentration of taurine (1 mM), expression was greater in *S. meliloti* 1021 than in *R. leguminosarum* Rlv3841 [2,643 versus 1,739 nmol min^{-1} (mg protein)$^{-1}$, respectively]. However, with a high concentration of taurine (10 mM), there was little difference in activity between *S. meliloti* 1021 and *R. leguminosarum* Rlv3841. This is likely to be due to the presence of a dedicated taurine uptake system in *S. meliloti* 1021, resulting in a higher intracellular concentration of taurine. In the absence of taurine, activity was indistinguishable from promoterless (pJP2) controls, demonstrating the high degree of promoter regulation. The available unique restriction sites for cloning downstream of *tauAp* are given in Fig. 1B; alternatively, additional sites within the *gusA* reporter gene can be utilized.

Host range of *tauR* and *tauAp*. To further investigate the suitability of pLMB51 as an expression vector for use in other bacteria, it was introduced into a number of *Proteobacteria*. Cells were grown in the presence of 10 mM taurine, and GusA activity was measured. No activity was detected in either *E. coli* or *Pseudomonas fluorescens* strain SBW25 (data not shown). However, activity

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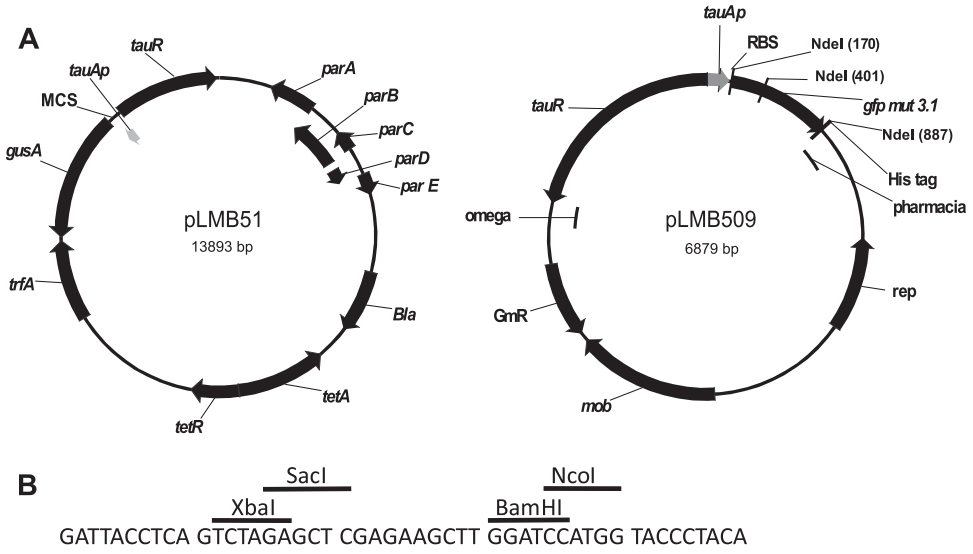

FIG 1 Taurine inducible vectors. (A) Maps of pLMB51 and pLMB509. (B) Multiple-cloning site (MCS) of pLMB51 showing unique restriction sites.

was detected in a range of *Alphaproteobacteria* (Fig. 4), including *Rhizobium etli* strain CE3, *Rhizobium* species strain NGR234 grown in AMS supplemented with 2 μg ml^{-1} tetracycline, 10 mM D-glucose, and 10 mM ammonium chloride, and *Rhodobacter sphaeroides* strain WS8N grown in TY containing 2 μg ml^{-1} tetracycline. This demonstrates the usefulness of pLMB51 as a broad-range alphaproteobacterial expression vector.

***In planta* expression.** *R. leguminosarum* Rlv3841 is a nitrogen-fixing symbiont that nodulates pea plants. To investigate if *tauAp* could be induced *in planta*, *R. leguminosarum* Rlv3841 carrying pLMB51 was inoculated on pea seeds (*Pisum sativum* cv. Avola) in 1-liter pots as described previously (1). Taurine was added to the growth medium as the plants matured on days 14 and 19. After 21 days, the nodules were crushed and bacteroids isolated as described previously (9), and GusA activity was determined (Fig. 5). Although activity was much weaker than in free-living cultures, significant expression was detected, even after 2 days of induction with as little as 1 mM taurine. This demonstrates the utility of pLMB51 as a vector for inducible *in planta* gene expression.

Highly inducible His tag expression vector pLMB509. Polyhistidine tagging of proteins (to the C or N terminus) provides an easy and routine method of protein purification using nickel affinity chromatography. Vector pLMB509 was constructed to utilize *tauAp* for the controllable overexpression of His-tagged proteins in *Alphaproteobacteria*. The vector is a derivative of pRU1097 (pBBR incompatibility) and was chosen because it has a higher copy number than pJP2. The vector pLMB509 incorporates a *gfpmut3.1* reporter gene downstream of *tauAp* and a ribosomal

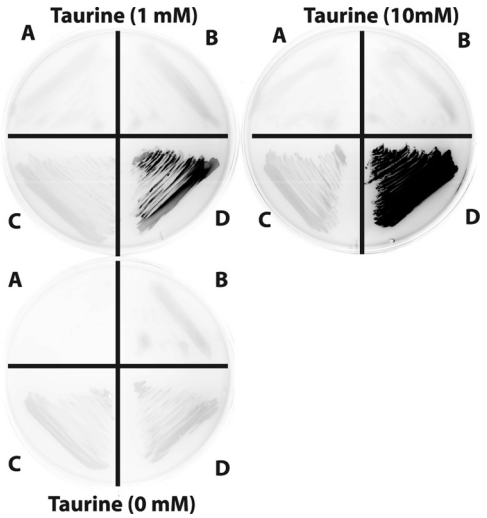

FIG 2 Green fluorescent protein (GFP) fluorescence of various fusions detected on a Fujifilm FLA-7000 image reader; darker shade indicates higher fluorescence. *R. leguminosarum* Rlv3841 and *S. meliloti* 1021 strains containing promoterless *gfpmut3.1* parent plasmid (pRU1097) or its derivative with *tauAp* upstream of *gfpmut3.1* (pRU1357), grown without (0 mM) or with (1 mM or 10 mM) taurine. Quadrant A, *R. leguminosarum* Rlv3841 pRU1097; quadrant B, *R. leguminosarum* Rlv3841 pRU1357; quadrant C, *S. meliloti* 1021 pRU1097; quadrant D, *S. meliloti* 1021 pRU1357.

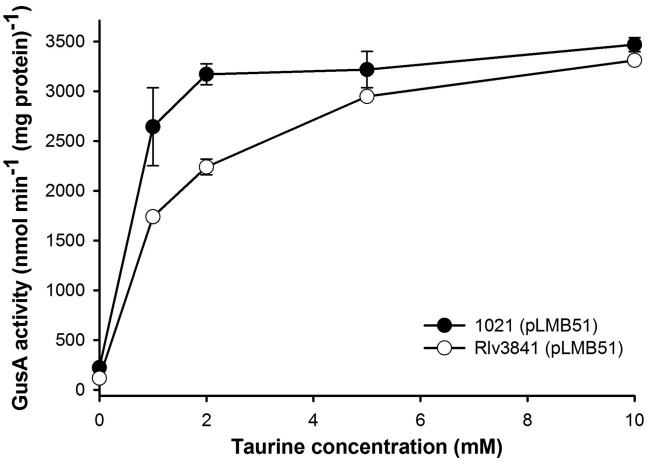

FIG 3 GusA activity of *R. leguminosarum* Rlv3841 and *S. meliloti* 1021 carrying pLMB51 when induced with different concentrations of taurine.

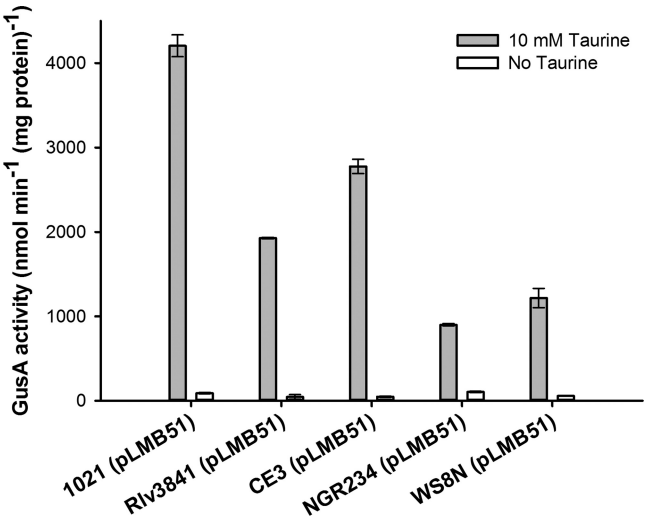

FIG 4 GusA activity of different *Alphaproteobacteria* carrying pLMB51 when induced with taurine.

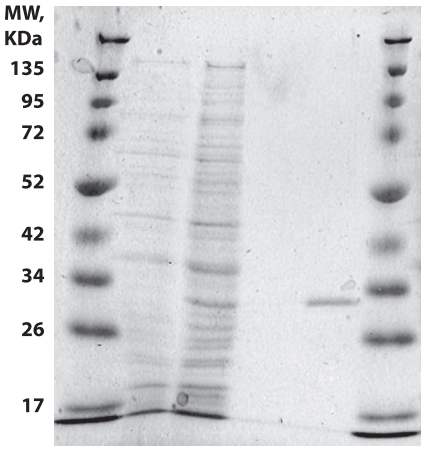

FIG 6 SDS-PAGE gel analysis of protein extractions. Lanes 1 and 6, protein ladder (Spectra; Fermentas). Lanes 2 and 4, total cell protein extraction and after nickel affinity chromatography purification are shown, respectively, when RLv3841 containing pLMB548 was uninduced and, in lanes 3 and 5, when induced with 10 mM taurine. The gel was stained with InstantBlue (Expedeon).

binding site (RBS), followed by a six-His tag and a transcriptional stop codon. NdeI restriction sites flank the reporter gene, enabling the easy removal of *gfpmut3.1* and subsequent replacement with the desired gene in frame (Fig. 1). When pLMB509 was conjugated into *R. leguminosarum* Rlv3841 and grown on TY medium, fluorescence was only detected when taurine was added to the medium (data not shown).

To further test the utility of the vector, *gfpmut3.1* was removed by NdeI restriction digest and substituted with a characterized fucose periplasmic solute binding protein from *S. meliloti* 1021 (SMc02774), including the native leader sequence, to give pLMB548. Although it would be possible to NdeI digest a gene for cloning into pLMB509, we utilized BD In-Fusion cloning (Clontech). BD is a rapid method for cloning PCR amplified products without the need for restriction digest, ligation, or polishing of blunt ends. This facilitates high-throughput gene cloning, including the cloning of genes containing NdeI restriction sites. Therefore, the fucose solute binding protein was PCR amplified using BD primers pr1172 (**AGGAGGAAGAACATATG**ATGAGCATA GCCAAGCGC) and pr1152 (**TGGTGATGATGCATATG**GTTG ACCTTCGGATTCAGAAG). The sequences in bold are complementary to the vector digested with NdeI and should be added to any gene-specific sequence for BD cloning into pLMB548. To extract the fucose SBP, *R. leguminosarum* Rlv3841 cells containing pLMB548 were grown to mid-log phase in AMS containing 10 mM D-glucose and 10 mM ammonium chloride with or without the addition of 10 mM taurine. Periplasmic extraction was as previously described (5). Histidine-tagged protein (SMc02774) was purified from the periplasmic extraction using a 1-ml HisTrap FF column at 4°C on an AKTA basic 10 system (GE Life Sciences) (Fig. 6). *R. leguminosarum* was chosen as the host rather than *S. meliloti* 1021 (from which *tauAp* and the SBP gene derives) to illustrate the ease of overexpressing and purifying a heterologous protein.

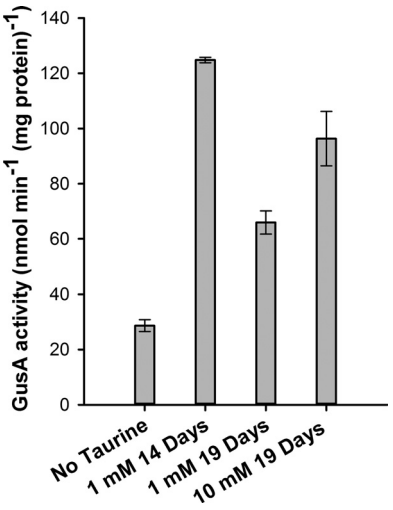

FIG 5 GusA activity of ground nodule extract after pea plants were induced with taurine after 14 and 19 days. Nodules were harvested after a total of 21 days of growth.

Vector uses. Expression vectors pLMB51 and pLMB509 have a low background and are highly inducible in a broad range of *Alphaproteobacteria*. Plasmid pLMB51 is a low-copy vector that is stably maintained without the need for continuous selection and, as such, is a useful tool for gene expression in bacteria either cultured in the laboratory or introduced into the environment. Plasmid pLMB509 has a higher copy number than pLMB51 and permits high-throughput cloning and expression of His-tagged proteins for purification. This can be very valuable for proteins derived from *Alphaproteobacteria* whose expression proves problematic in hosts such as *E. coli.* Taurine is readily available and is less expensive than IPTG (isopropyl-β-D-thiogalactopyranoside), making it an excellent inducer.

Nucleotide sequence accession numbers. The pLMB51 and pLMB509 sequences have been deposited in GenBank, National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), under the accession numbers JQ895026 and JQ895027, respectively.

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