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Tightly Regulated Gene Expression System in *Salmonella enterica* Serovar Typhimurium

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A new *Salmonella enterica* serovar Typhimurium strain has been constructed to facilitate tightly regulated gene expression. Arabinose-inducible and glucose-repressible expression of a T7 RNA polymerase gene that has been integrated with an adjacent *araC*-P_{BAD} control element into the bacterial chromosome allows dynamic control of T7 promoter-driven RNA transcription.

We have designed a new *Salmonella enterica* serovar Typhimurium construct to emulate the *Escherichia coli* BL21(DE3) system (16, 17). The construct allows for improved control of gene expression, particularly in terms of decreasing induction-independent leaky expression (2). This *Salmonella* construct has stable, site-specific chromosomal integration of the *araC*-P_{BAD} control element (6) in tandem with the T7 RNA polymerase gene (12). The arabinose *araC*-P_{BAD} element allows for quite exact control of T7 RNA polymerase expression. The T7 RNA polymerase in turn transcribes genes under T7 promoter control, including those encoding external guide sequences (EGSs) (4). EGSs are oligoribonucleotides that are used to target a complementary mRNA for cleavage by RNase P in vitro or in vivo (4) in *E. coli* (5, 10). This new construct provides a general expression platform for tightly controlled T7 promoter-driven gene transcription in *Salmonella* and, in particular, allows for studies of EGSs in this bacterium.

Two plasmids were produced for the controlled expression of T7 RNA polymerase: one for chromosomal integration (pSBaadABADT7-1) and one for maintenance as a replicating plasmid (pACBADT7-1). Performance data for the latter are available on request. Our plasmid nomenclature employs the abbreviations BAD for the *araC*-P_{BAD} control element (6) and T7-1 for T7 RNA polymerase (12). Figure 1 shows details of plasmid construction. The pSBaadABADT7-1 plasmid contains one copy of the *araC*-P_{BAD}-T7*pol* genes, flanked by *aadA* sequences, in a pSB890 backbone (8).

The new *Salmonella* construct, SB300A#1, was produced from the *Salmonella* parent strain SB300 (8) by using homologous recombination techniques (7) in which *E. coli* SM10λpir (15) serves as a conjugation partner that donates pSBaadABADT7-1 to SB300. Key components of pSBaadABADT7-1 include *tet, oriR6K* (7, 11), *sacB* (7), *aadA* (7, 9), *araC*-P_{BAD} (6), and the T7 RNA polymerase gene (12). The *aadA* gene, encoding a streptomycin adenylyltransferase which can mediate streptomycin and spectinomycin resistance, serves as the site of recombination (7, 9). Step-by-step details of our selection strategy for SB300A#1 are available on request. Briefly, we first selected for tetracycline resistance and then against sucrose sensitivity of the *tet* and *sacB* components, respectively, of pSBaadABADT7-1. This method is directly analogous to the approach described previously (7; Fig. 2 of this reference describes the genetics of the homologous recombination). Other selection maneuvers of potential utility for the pSBaadABADT7-1 system include screening for the loss of spectinomycin resistance (9) after homologous recombination into *aadA* (SB300A#1 loses the spectinomycin resistance exhibited by SB300 [data not shown]) and/or selecting for gain and subsequent loss (1) of tetracycline resistance.

The *araC*-P_{BAD} and T7 RNA polymerase components (not previously used in pSB890-based systems) were assessed in several ways. Portions (totaling ~250 bases at the 5′ end [data not shown]) of the T7 RNA polymerase gene were sequenced, and all were as per the reported sequence (12). An appropriately sized protein (~99 kDa) for T7 RNA polymerase was produced, in an arabinose-inducible manner, in *E. coli* cells that had been transformed with pSBaadABADT7-1 (Fig. 2A). In all experiments using metabolic regulators, a 0.2% concentration of arabinose or glucose in Luria-Bertani medium was used unless otherwise specified. Culture incubation conditions were as previously described (10). We will supply complete information regarding experimental methods on request.

The arabinose-inducible T7 RNA polymerase expression product recovered from 2 μl of crude cell extracts of either *E. coli* or *Salmonella* serovar Typhimurium transformants was shown to have functional enzymatic activity in the transcription in vitro of 2 μg of the DNA template from plasmid pBSC5 (3) under T7 promoter control (Fig. 2B). PCR analysis was used to demonstrate the new acquisition of the T7 RNA polymerase gene by *Salmonella* strain SB300 after either transformation of cells (13) with relevant plasmids or selection of cells based on the stable integration of the gene into the chromosome of SB300 (Fig. 2C). For PCRs, we used T7NcoF (ACCATGGG GAACACGATTAACATCGC) and T7HIIIR (CCGAAGCT TACGCGAACGCGAAG) as primers and 25 cycles of 94, 37, and 72°C. A total of four stable integration candidates (two are shown in Fig. 2C) were screened via PCR, with two candidates

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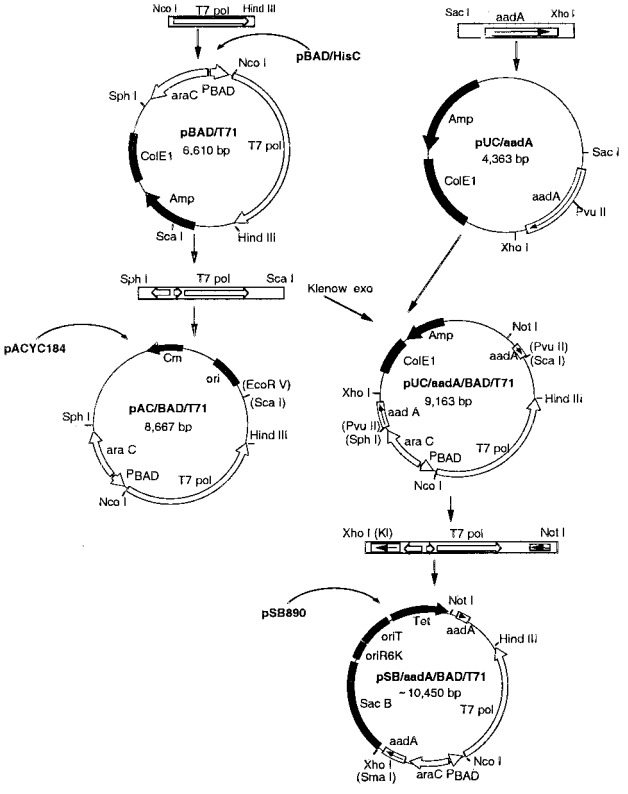

FIG. 1. Schematic view of vector construction. Maps of the arabinose-inducible T7 RNA polymerase expression plasmids pSB/aadA/BADT7-1 and pAC/BADT7-1 and their construction intermediates are shown with relevant features. Products of the serial steps of construction are shown at the confluence of schematic arrows leading from that product's constituent parts: for example, pBAD/T7-1 results from the cloning of the *Nco*I-*Hin*dIII-delimited fragment containing T7*pol* into the pBAD/HisC plasmid. Plasmid names are in bold type, including those of vectors denoted by name alone (pBAD/HisC, pACYC184, and pSB890), which provided backbones for subsequent constructs. Cloned fragments used as inserts for construction steps are represented as ribbons that contain arrows denoting key functional components and their 5′–3′ orientations. The *aadA* gene in pUC/aadA is interrupted (by insertion into its *Pvu*II site) during the construction of pUC/aadA/BADT7-1, and hence *aadA* is subsequently represented as two interrupted parts. Pertinent restriction sites are provided; restriction sites listed in parentheses [such as (*Sma*I) in pSB/aadA/BADT7-1] are destroyed by the cloning process. *Xho*I (Kl) is an *Xho*I site made blunt by the Klenow fragment.

(including SB300A#1) being positive and two (including SB300A#14) being negative.

The control in vivo and the activity of the integrated T7 RNA polymerase in SB300A#1 were assessed by using Northern blotting to detect a T7 promoter-driven RNA strand, transcribed from the plasmid pUC19 M1:AEF2HH (C. Guerrier-Takada, unpublished data), which encodes the M1 RNA

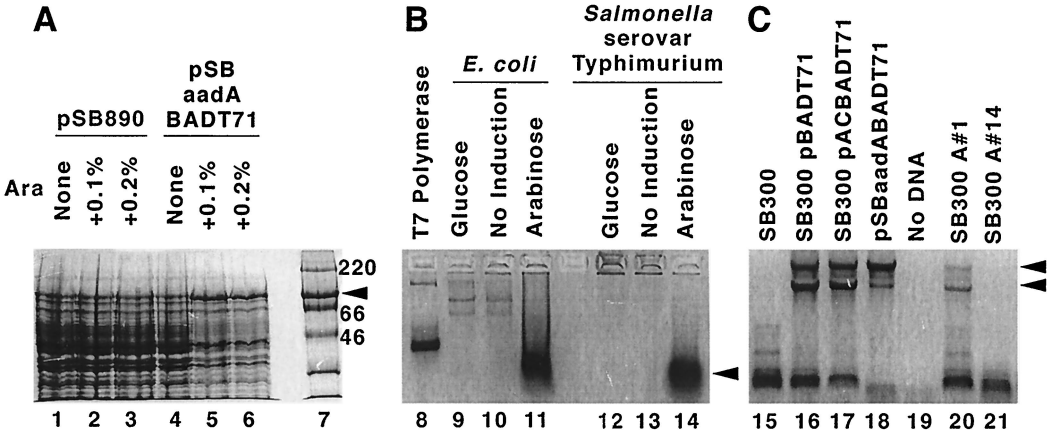

FIG. 2. Assessment of intermediate construction steps. (A) Expression of presumptive T7 RNA polymerase after induction with arabinose. Protein expression is shown for crude cell extracts of *E. coli* which were transformed either with pSB890 (lanes 1 to 3) or with pSBaadABADT7-1 (lanes 4 to 6) and then exposed during growth in liquid culture to the indicated arabinose concentrations. Lane 7 provides molecular mass markers: a 97.4-kDa band is left of the arrowhead. For lanes 5 and 6, the arrowhead denotes the expression of a protein of appropriate size (~99 kDa) for T7 RNA polymerase. This expression occurs after induction with arabinose in *E. coli* transformed with pSBaadABADT7-1 (lanes 4, 5, and 6) but not in pSB890 transformants (lanes 1, 2, and 3), which lack the *araC* control element and the T7 RNA polymerase gene. (B) In vitro enzymatic activity of T7 RNA polymerase that was recovered from crude cell extracts of bacterial transformants. T7 RNA polymerase activity was assessed by detection of RNA (denoted by the arrowhead) with an ethidium bromide agarose gel, resulting from transcription in vitro of DNA behind a T7 promoter. Purified recombinant T7 RNA polymerase was used as a positive control (lane 8). Other specimens are crude cell extracts of *E. coli* (lanes 9, 10, and 11) or *Salmonella* serovar Typhimurium (lanes 12, 13, and 14) transformants. Both *E. coli* and *Salmonella* serovar Typhimurium contain plasmids encoding T7 RNA polymerase and the *araC* control element and either were subjected in liquid culture to treatment with glucose (for repression) or arabinose (for induction) or were not induced, as shown. *E. coli* cells were transformed with pSBaadABADT7-1 and were used as donors for subsequent conjugation. *Salmonella* serovar Typhimurium cells were transformed with pBADT7, an intermediate plasmid in the construction of pSBaadABADT7-1 (Fig. 1). (C) Detection of the T7 RNA polymerase gene in bacterial transformants and conjugation products by using PCR amplification. Arrowheads indicate PCR products corresponding with the presence of the T7 RNA polymerase gene. PCR amplification of the T7 RNA polymerase gene from plasmid or genomic DNA is shown for various *Salmonella* serovar Typhimurium constructs. Lane 15 is a negative control, the parent *Salmonella* serovar Typhimurium strain (SB300) prior to manipulation. Lanes 16 and 17 are *Salmonella* serovar Typhimurium transformants containing the T7 RNA polymerase gene in the plasmids pBADT7-1 and pACBADT7-1 (Fig. 1). Lane 18 is a large-scale plasmid preparation of pSBaadABADT7-1 from an *E. coli* transformant. Lane 19 is the PCR mixture without DNA. Lanes 20 and 21 are *Salmonella* serovar Typhimurium strains recovered after the selection and screening steps described in the text. The strains in lanes 20 (denoted SB300 A#1) and 21 (denoted SB300 A#14) are with and without a PCR-detectable T7 RNA polymerase gene, respectively. Both SB300A#1 and SB300A#14 were used for the in vivo experiment described in the legend for Fig. 3; SB300A#1 was used for the experiments reported in the legend for Fig. 4.

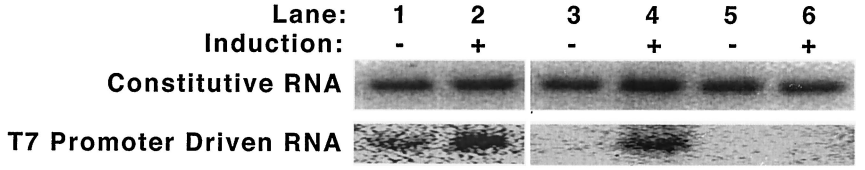

FIG. 3. Basal and induced gene expression in *E. coli* BL21(DE3) and the new *Salmonella* serovar Typhimurium construct. Northern blots of constitutive and induced RNA transcription are shown for two systems: *E. coli lac*-based induction with IPTG (lanes 1 and 2) and the new *Salmonella* serovar Typhimurium system, *ara*-based induction with arabinose (lanes 3 to 6). The *Salmonella* serovar Typhimurium in lanes 3 and 4 (SB300A#1) has a PCR-detectable T7 RNA polymerase gene in genomic DNA, whereas the *Salmonella* serovar Typhimurium in lanes 5 and 6 (SB300A#14) lacks an integrated T7 RNA polymerase gene (Fig. 2C). Note the leaky transcription of T7 promoter-driven RNA even without induction in the *E. coli lac* system, in contrast to the off-on expression in the new *Salmonella* serovar Typhimurium *ara* system. Levels of constitutive RNA expression (assessed here by Northern blotting for the M1 RNA component of RNase P) are grossly similar in all samples, which were total RNA extracts from bacteria pelleted from liquid cultures.

component of RNase P (14) fused with a "three-quarter" form EGS (4). In cell extracts of cultures (grown for 1 h with or without induction) containing similar amounts of constitutively expressed RNA, *E. coli* BL21(DE3) exhibits leaky basal expression of a T7 promoter-driven RNA even without induction with IPTG (isopropyl-β-D-thiogalactopyranoside) (Fig. 3). By contrast, in the new *Salmonella* serovar Typhimurium construct SB300A#1, leaky expression of the T7 promoter-driven RNA is not detected. Expression is seen in SB300A#1 after induction with arabinose (Fig. 3) but not in SB300A#14, which lacks integrated T7 RNA polymerase (Fig. 2 and 3).

The kinetics of T7 promoter-driven RNA expression in SB300A#1 are shown in Fig. 4. The results are normalized to constitutively expressed 5S RNA. Induced RNA expression is detected within 20 min of arabinose induction of SB300A#1 liquid cultures. This RNA shows an increased level of expression from 1 to 2.5 h after induction, and this level decreases by 19 h after induction. This induced expression can be modulated, as shown when glucose was added to a liquid culture of SB300A#1 bacteria, which was pelleted and washed after 20 min of induction with arabinose. Arabinose-induced T7 promoter-driven RNA expression was repressed to the background levels seen in uninduced bacteria within 60 to 90 min of the addition of glucose. Resuspension of the same arabinose-induced cultures in glucose-free medium does not inhibit expression of T7 promoter-driven RNA at 90 min (data not shown).

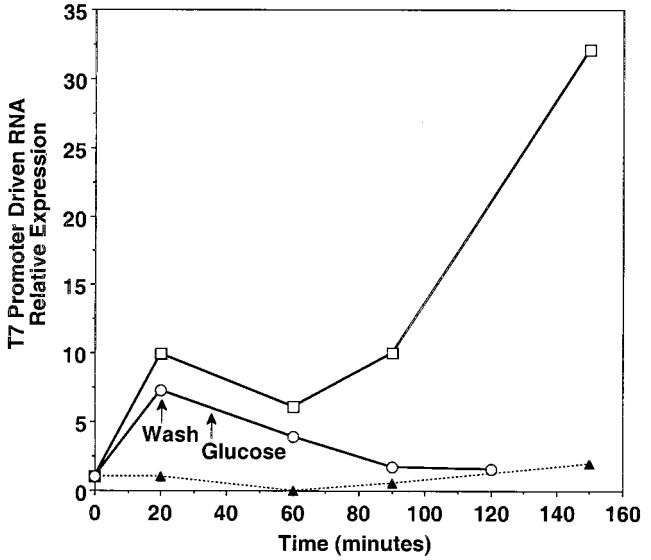

FIG. 4. Kinetics of gene expression control. Quantitation of on and off gene expression after induction with arabinose and repression with glucose. Northern blot signals of induced T7 promoter-driven RNA expression, normalized to constitutive RNA expression, are shown over time. Specimens are total RNA extracts of *Salmonella* serovar Typhimurium bacteria pelleted from liquid cultures at various times after the addition of the inducing agent arabinose (□), with arabinose induction followed by washing and by the addition of glucose (○), or without the addition of arabinose (▲).

The dynamic response in gene expression observed with this system was not guaranteed. While the *araC* control element does exhibit a clear distinction between induction and repression, the T7 RNA polymerase expressed under *araC* control must subsequently transcribe the gene of interest. The induced T7 RNA polymerase appears to have net effects on transcription which peak and then decrease rapidly. Furthermore, the reporter RNA we measure as an output of our control system likely has a short survival half-life in the cell. Together, these features prevent the system from becoming overly damped. That is, despite a series of required steps between the addition of arabinose (or glucose) and the expression (or repression) of the gene product of interest, the transition between on and off expression states remains clear.

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