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Construction and Use of a Versatile Set of Broad-Host-Range Cloning and Expression Vectors Based on the RK2 Replicon

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The plasmid vectors described in this report are derived from the broad-host-range RK2 replicon and can be maintained in many gram-negative bacterial species. The complete nucleotide sequences of all of the cloning and expression vectors are known. Important characteristics of the cloning vectors are as follows: a size range of 4.8 to 7.1 kb, unique cloning sites, different antibiotic resistance markers for selection of plasmid-containing cells, *oriT*-mediated conjugative plasmid transfer, plasmid stabilization functions, and a means for a simple method for modification of plasmid copy number. Expression vectors were constructed by insertion of the inducible *Pu* or *Pm* promoter together with its regulatory gene *xylR* or *xylS*, respectively, from the TOL plasmid of *Pseudomonas putida*. One of these vectors was used in an analysis of the correlation between phosphoglucomutase activity and amylose accumulation in *Escherichia coli*. The experiments showed that amylose synthesis was only marginally affected by the level of basal expression from the *Pm* promoter of the *Acetobacter xylinum* phosphoglucomutase gene (*celB*). In contrast, amylose accumulation was strongly reduced when transcription from *Pm* was induced. CelB was also expressed with a very high induction ratio in *Xanthomonas campestris*. These experiments showed that the *A. xylinum celB* gene could not complement the role of the bifunctional *X. campestris* phosphoglucomutase-phosphomannomutase gene in xanthan biosynthesis. We believe that the vectors described here are useful for cloning experiments, gene expression, and physiological studies with a wide range of bacteria and presumably also for analysis of gene transfer in the environment.

The use of nonenteric bacteria for basic and applied molecular research has extended the need for well-characterized vector systems for such organisms. In practice these problems are solved either by developing specific vector systems for each species of interest or by taking advantage of already-available broad-host-range replicons. The latter approach has the advantage that a few such systems can be studied more extensively, and the accumulated knowledge can then be utilized with many species. Vectors based on the broad-host-range RK2 and RSF1010 replicons represent the most frequently used systems (19), but the derivatives in common use (for examples, see references 42, 45, 49, and 51) have not been developed to a level of sophistication comparable to that for standard *Escherichia coli* vectors.

RSF1010 and RK2 belong to different incompatibility groups, and these replicons can therefore be maintained together in the same cell. RK2 is a 60-kb self-transmissible plasmid, and its complete nucleotide sequence has been reported (52). The replicon is known to be functional in 29 (and probably many more) gram-negative bacterial species (for reviews, see references 25, 67, and 68) and was later also reported to replicate in the gram-positive organism *Clavibacter xyli* (47) and in the gram-negative organisms *Bartonella bacilliformis* (24) and *Actinobacillus actinomycetemcomitans* (23). The minimal RK2 replicon consists of the origin of vegetative replication (*oriV*) and the gene encoding an essential initiator protein (TrfA) that binds to iterons in *oriV* (53–55). The *trfA* gene specifies two protein products of 44 and 33 kDa, and these two proteins originate from alternative translational starts within the same open reading frame (64).

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The copy number of RK2 is estimated to be 5 to 7 per chromosome in *E. coli* (16). Certain point mutations in the *trfA* gene have been shown to specify elevated (up to 24-fold) copy numbers of minimal RK2 replicons, and *trfA* mutants that are temperature sensitive for replication have also been reported (8, 13, 28, 29, 69). Copy-up mutants isolated from *E. coli* are also copy up in other bacteria (and vice versa), but many species seem to tolerate a rather low number of RK2 replicons per cell (29). All characterized copy-up mutants have been localized between the *Sfi*I and *Nde*I sites in *trfA*, and this can be utilized to modify the copy number of the RK2 plasmid vectors described in this report.

RK2 encodes two operons containing the *parDE* and *parCBA* genes, respectively, which are involved in the maintenance of RK2 plasmids or heterologous replicons in diverse bacterial populations (59, 60, 63). This is in contrast to the *par* system from the narrow-host-range plasmid F (59). It has recently been reported that the importance of the functions encoded by *parCBA* and *parDE* varies from host to host (65).

In this paper we describe the construction of a series of well-characterized, broad-host-range, multipurpose cloning vectors based on the RK2 replicon. These vectors were designed to simplify routine experiments in molecular biology with bacteria. We also wanted to develop tightly controlled gene expression systems, and for this purpose we used the *Pu* and *Pm* promoters and the corresponding positive regulatory genes *xylR* and *xylS*, all originating from the TOL plasmid of *Pseudomonas putida* (22; for reviews, see references 4, 27, and 44). These expression systems are known to function in a wide variety of gram-negative bacteria (36, 46, 57).

To characterize the functionalities of the two promoters, we used the gene encoding the enzyme phosphoglucomutase (CelB) from *Acetobacter xylinum* (17). The broad-host-range

properties of this expression system were confirmed by studying *celB* expression in *Xanthomonas campestris.*

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are described in Table 1. *Pseudomonas aeruginosa* and *E. coli* strains were grown in L broth or on L agar (61). In the amylose accumulation experiments L broth was supplemented with 1% maltose. The growth temperature was 30°C for *P. aeruginosa. E. coli* cells were grown at 37°C, except for the expression analysis of *celB* transcribed from the *Pm* and *Pu* promoters, where 30°C was used. *Azotobacter vinelandii* and *X. campestris* were grown at 30°C in Burk medium (63) and YM broth (Difco), respectively. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; carbenicillin, 100 μg/ml; tetracycline, 15 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 50 μg/ml; and streptomycin, 2 mg/ml.

Conjugative matings and electrotransformations. Conjugative matings from *E. coli* to *P. aeruginosa* were performed on membranes, and the mixtures were incubated on nonselective agar medium at 30°C for 3 h. S17.1 containing the relevant plasmids was used as a donor strain. The mating mixture was incubated for 3 h at 30°C and then plated on agar medium containing carbenicillin and streptomycin. Plasmids were transferred to *A. vinelandii* and *X. campestris* by electrotransformation at a field strength of 12.5 kV/cm, as described for *E. coli* (26), and the cells were then plated on agar medium containing ampicillin.

DNA manipulations. Plasmid DNA was prepared by the alkaline lysis protocol for *E. coli*, and all other standard techniques were performed as described by Sambrook et al. (61). When needed, restriction enzyme sites were made blunt by the use of either Klenow (5′ protruding ends) or T4 DNA polymerase (3′ protruding ends). The following linkers were used for the conversion of enzyme sites: *Bgl*II 5′ d(pCAGATCTG), *Eco*RI 5′d(pGGAATTCC), *Hin*dIII 5′ dp(CA AGCTTG), *Nsi*I 5′ dp(CATGCATG), and *Xba*I 5′ dp(CTCTAGAG). Transformations of *E. coli* were performed by the method of Chung et al. (9). DNA sequencing was performed by the dideoxy chain termination method (62). Cell growth (optical density at 600 nm [OD_{660}]) was monitored with a Beckman DU-65 spectrophotometer.

Analysis of plasmid stability. *E. coli* DH5α, *A. vinelandii*, and *P. aeruginosa* containing pJB3*E* or pJB321*E* were grown under selection to stationary phase, diluted 100-fold in the same medium, and then grown exponentially under selection. The stability assay was initiated by dilution of the cells to 10^3 cells/ml in nonselective medium, followed by growth overnight. Cultures were then again diluted and grown overnight in nonselective medium (as described above), and this procedure was repeated until the total number of generations had reached 200 to 400, as indicated in Results. After each dilution, aliquots were plated on nonselective agar medium. The colonies were sprayed with 50 mM catechol to identify plasmid-containing cells as yellow colonies (18), and yellow colonies were double-checked by transfer to agar medium containing ampicillin. Yellow colonies were always ampicillin resistant, while white colonies were not.

Expression studies and amylose measurements. Preparation of cell extracts and measurements of phosphoglucomutase activities were performed as described by Fjærvik et al. (17). Amylose accumulation was measured as previously described (6).

Nucleotide sequence accession numbers. The sequences of the following vectors have been deposited in the GenBank database with the indicated accession numbers: pJB3, U73899; pJB3Cm6, U75322; pJB3Km1, U75323; pJB3Tc20, U75324; pJB321, U75325; pJB653, U75327; and pJB137, U75326.

RESULTS

Construction of general-purpose broad-host-range cloning vectors. Figure 1 outlines the procedures used to construct a set of relatively small RK2-based vectors with different antibiotic resistance markers (pJB3, Ap^r; pJB3Cm6, Ap^r Cm^r; pJB3Tc20, Ap^r Tc^r; and pJB3Km1, Ap^r Km^r). Plasmid pFF1 was used as a starting point for all of the constructs, and many of the steps in the construction procedure served to delete unnecessary DNA sequences (size reduction), to eliminate undesired restriction endonuclease sites, or to create new sites. One of the useful consequences of this was that the *Nde*I and *Sfi*I sites in *trfA* were kept unique. Since all known copy-up mutations in this gene are localized between these two sites, the copy numbers of all of the vectors can be modified by straightforward one-step subcloning procedures (29). All vectors share a common polylinker and *lacZ′* region, simplifying standard cloning procedures and identification of plasmids with inserts by blue-white screening in *E. coli*. Most of the restriction endonuclease sites in the polylinker region are unique, and the exceptions are caused by the presence of some of these sites in antibiotic resistance genes. All vectors contain *oriT* (origin of transfer), which is useful in cases where transformation or electroporation is inefficient or if very high transfer frequencies are required.

The complete nucleotide sequences of the vectors were established by combining sequences previously reported in the literature and by sequencing many of the junction sites involved in the construction procedures. This greatly simplifies the routine use of the vectors, further improvements, and generation of more-specialized derivatives.

Vector stability. In some hosts, plasmid stability may not be satisfactory, and we therefore inserted *parDE* into pJB3, generating pJB321, as shown in Fig. 1. To simplify stability measurements, the *xylE′* fragment from pJB109 was also inserted in the polylinker of pJB3 and in pJB321, generating plasmids pJB3*E* and pJB321*E*, respectively. The fragment was inserted in such an orientation that *xylE′* could be transcribed from the *lac* promoter in the vector. Figure 2 demonstrates the stabilizing effects of the *parDE* sequences in three different species. In *E. coli* the unmodified plasmid (pJB3*E*) is relatively stable, but in the presence of *parDE* (pJB321*E*) virtually no plasmid loss was observed (Fig. 2A). It has been previously reported that in *A. vinelandii* an RK2 derivative was very unstable but could be stabilized by *parDE* (60, 65). As can be seen from Fig. 2B, pJB321*E* is much more stable than pJB3*E*, illustrating the usefulness of this vector modification for certain hosts. In *P. aeruginosa*, the stability difference between the two plasmids was marginal (Fig. 2C), but the frequency of plasmid loss was so low in both cases that for most purposes practical problems should not be experienced.

Construction of broad-host-range expression vectors. Plasmid pJB7 was used as a starting point for the construction of expression vectors pJB137 and pJB653, containing the *Pu* and *Pm* promoters, respectively (Fig. 3). In the first steps, the genes encoding the positive regulators XylR and XylSArg41Pro were inserted. The mutant gene *xylS839*, encoding XylSArg41Pro, was used because it causes a reduction of the basal transcription level from *Pm*, compared to that with wild-type *xylS* (48). The *Pu* and *Pm* promoters were then inserted, generating plasmids pJB134 and pJB64, respectively. The remaining steps up to the final constructs, pJB137 and pJB653, served to fill in undesired restriction endonuclease sites, to create new sites, and to insert a bidirectional transcriptional terminator between the *Pu* or *Pm* promoter and the *trfA* gene. This terminator has previously been shown to function in a wide variety of gram-negative species (14, 20). In this way, readthrough transcription from the *trfA* gene into the *Pu* and *Pm* promoters is prevented, and transcription initiated at *Pu* or *Pm* should not affect TrfA expression. To simplify the routine use of these expression vectors, they contain a polylinker region downstream of the *Pu* and *Pm* promoters (Fig. 3). In analogy to pJB321 (Fig. 1), the *parDE* region was also inserted into each of the constructs, generating pJB139 and pJB654 (Table 1).

Expression of the *A. xylinum* phosphoglucomutase gene, *celB*, from the *Pu* and *Pm* promoters. The 1.9-kb *Bam*HI *celB* fragment from pUC7*celB* was cloned in an orientation that allowed transcription of the gene from *Pu* in pJB137 and *Pm* in pJB653, generating pJB137*celB* and pJB653*celB*, respectively. The expression levels were then monitored as a function of cell growth (Fig. 4A). As can be seen, the *Pu* promoter expresses very low levels of phosphoglucomutase in the absence of inducer as long as the cells are kept growing exponentially. The expression level in the presence of the inducer is also low but is severalfold higher than that in uninduced cells. As the cells enter stationary phase, the expression levels in the uninduced

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Properties[a]	Source or reference
Strains		
E. coli		
DH5α	*endA1 hsdR17 supE44 thi-1* λ⁻ *recA1 gyrA96 relA1* Δ*lac*U169 (ϕ80d*lacZ*ΔM15)	Bethesda Research Laboratories
S17.1	RP4 2-Tc::Mu-Km::Tn7 *pro res mod*⁺	66
PGM1	*pgm* derivative of Hfr3000	3
P. aeruginosa PAO1161S	Spontaneous streptomycin-resistant derivative of PAO1161	29
A. vinelandii UW	Wild type	5
X. campestris B100-152	Spontaneous *xanA* exopolysaccharide-negative mutant	31
Plasmids		
RK2	60-kb broad-host-range plasmid originally isolated from *Klebsiella aerogenes*; Ap^r Km^r Tc^r	33
pFF1	RK2 minimal replicon; Ap^r Cm^r; 5.9 kb	13
pJB2	Derivative of pFF1 in which the *Eco*RI, *Bgl*II, and *Sal*I sites were filled in by three steps; Ap^r Cm^r; 5.9 kb	This work
pUC19	ColE1 replicon; Ap^r; 2.7 kb	50
pUC19-3	Derivative of pUC19 in which the *Nde*I site was filled in (step 1) and the *Ssp*I and *Afl*III sites flanking the *lac* region were converted to *Nsi*I and *Bgl*II (steps 2 and 3, respectively); Ap^r; 2.7 kb	This work
pJB5	Derivative of pJB2 in which 0.5 kb of the upstream part of the Cm^r gene was deleted with *Pvu*II digestion followed by insertion of a *Bgl*II linker at the same site (step 1) and then two *Bam*HI sites flanking *Pneo* were also filled in (step 2); Ap^r; 5.4 kb	This work
pKH3	Derivative of pJB5 in which the 0.7-kb *Pst*I/*Bgl*II fragment was replaced with a 1.0-kb *Nsi*I/*Bgl*II fragment containing the polylinker and *lac* regions from pUC19-3; Ap^r; 5.7 kb	This work
pJB7	Deletion derivative of pJB5 obtained by digestion with *Afl*III plus *Eco*47-3 (0.4 kb, step 1) and by *Not*I plus partial *Acc*I digestion (0.5 kb, step 2); Ap^r; 4.5 kb	This work
pJB3	Derivative of pJB7 in which the 1.5-kb *Bgl*II/*Sfi*I fragment was replaced with a 1.8-kb *Bgl*II/*Sfi*I fragment containing the polylinker and *lac* regions from pKH3; Ap^r; 4.8 kb	This work
pRR120	pBluescript II SK(+) with 0.8-kb *parDE* region from RK2; Ap^r; 3.8 kb	60
pJB9	Derivative of pRR120 in which the polylinker sites between *Hin*dIII and *Sma*I, downstream of *parDE*, were deleted by digestion with *Hin*dIII (filled in) and *Sma*I; Ap^r; 3.8 kb	This work
pJB10	Derivative of pJB9 in which the *Kpn*I site upstream of *parDE* was converted to *Bgl*II; Ap^r; 3.8 kb	This work
pHL12	Derivative of pJB9 in which the *Bam*HI site downstream of *parDE* was filled in (step 1) and the *Kpn*I site upstream of *parDE* was converted to *Xba*I (step 2); Ap^r; 3.8 kb	This work
pJB313	Derivative of pJB3 in which the 0.8-kb *Bgl*II/*Bam*HI fragment containing the *parDE* fragment from pJB10 was inserted into the *Bgl*II site; Ap^r; 5.6 kb	This work
pJB321	Same as pJB313 except that the *parDE* fragment is in the opposite orientation	This work
pα*xylE*Ω	RSF1010 replicon; Cm^r; 13.2 kb	21
pUC7	ColE1 replicon; Ap^r; 2.7 kb	70
pJB107	Derivative of pUC7 in which the promoterless *xylE* gene from pα*xylE*Ω was cloned as a 2.0-kb *Bam*HI fragment into pUC7 digested with the same enzyme; Ap^r; 4.7 kb	This work
pJB109	Derivative of pJB107 in which the two *Sac*II sites flanking the *xylE* gene in pJB107 were converted to *Eco*RI sites (step 1) and this 1.2-kb *Eco*RI fragment (here designated *xylE′*) was then cloned into pUC7 digested with *Eco*RI (step 2); Ap^r; 3.9 kb	This work
pJB3*E*	Derivative of pJB3 in which the 1.2-kb *Eco*RI *xylE′* fragment from pJB109 was cloned into the polylinker *Eco*RI site in pJB3; Ap^r; 6.0 kb	This work
pJB321*E*	Derivative of pJB321 in which the 1.2-kb *Eco*RI *xylE′* fragment from pJB109 was cloned into the polylinker *Eco*RI site in pJB321; Ap^r; 6.8 kb	This work
pSV16	RK2 replicon; Ap^r Km^r; 3.3 kb	69
pUC128	ColE1 replicon; Ap^r; 3.2 kb	35
pUC128Km	Derivative of pUC128 in which the Km^r gene from pSV16 was cloned as a 1.2-kb *Bam*HI fragment into the *Bam*HI site of pUC128; Ap^r; 4.4 kb	This work
pJB3Km1	Derivative of pJB3 in which the Km^r gene of pUC128Km was inserted into the *Bgl*II site as a 1.2-kb *Bam*HI fragment; Ap^r Km^r; 6.1 kb	This work
pJB3Km2	Same as pJB3Km1 except that the Km^r gene was cloned in the opposite orientation	This work
pUC7Tc	Derivative of pUC7 in which the Tc^r gene of RK2 was cloned as a 2.3-kb blunt-ended *Stu*I/*Bgl*II fragment into the *Hin*cII site of pUC7; Ap^r Tc^r; 4.9 kb	This work
pJB3Tc20	Derivative of pJB3 in which the Tc^r gene from pUC7Tc was inserted as a 2.3-kb *Bam*HI fragment into the *Bgl*II site; Ap^r Tc^r; 7.1 kb	This work
pJB3Tc19	Same as pJBTc20, except that the Tc^r gene was cloned in the opposite orientation	This work
pUC7Cm	Derivative of pUC7 in which the Cm^r gene was cloned as a 1.4-kb *Pst*I/*Hgi*AI blunt-ended fragment from pFF1 into the *Hin*cII site of pUC7 (the *Hgi*AI site is localized proximal and counterclockwise to *oriT*); Ap^r Cm^r; 4.1 kb	This work
pJB3Cm6	Derivative of pJB3 in which the Cm^r gene of pUC7Cm was cloned as a 1.4-kb *Bam*HI fragment into the *Bgl*II site; Ap^r Cm^r; 6.2 kb	This work

Continued on following page

TABLE 1—*Continued*

Bacterial strain or plasmid	Properties[a]	Source or reference
pJB3Cm10	Same as pJB3Cm6 except that the Cm^r gene was cloned in the opposite orientation	This work
pJB8	Derivative of pJB7 in which the *Nco*I site was converted to *Eco*RI; Ap^r; 4.5 kb	This work
pERD839	RSF1010 replicon containing *xylS839*; Km^r Sm^r; 14.7 kb	48
pJB86	Derivative of pJB8 in which *xylS839* was cloned as a 1.6-kb *Bam*HI fragment from pERD839 into the *Bgl*II site; Ap^r; 6.1 kb	This work
pERD21	RSF1010 replicon containing the *Pm* promoter; Km^r; 13.8 kb	57
pUC129	ColE1 replicon; Ap^r; 3.3 kb	35
pJB103	pUC129 with the *Pm* promoter cloned as an 0.7-kb *Eco*RI/*Pvu*II fragment from pERD21 into the *Eco*RI/*Eco*RV-digested vector; Ap^r; 4.0 kb	This work
pJB64	Derivative of pJB86 in which the *Pm* promoter was cloned as a 0.7-kb *Nsi*I/*Eco*RI fragment from pJB103 into pJB86 digested with *Pst*I (same overhang as *Nsi*I) and *Eco*RI; Ap^r; 6.4 kb	This work
pJB651	Derivative of pJB64 in which the orientation of *Pm* was reversed by digestion with *Kpn*I followed by religation (step 1), a series of restriction endonuclease sites upstream of *Pm* were eliminated by *Hin*dIII and *Eco*RI digestion (step 2) and downstream of *Pm* by *Sal*I and *Bam*HI digestion (step 3), and the remaining *Kpn*I site downstream of *Pm* was converted to a *Hin*dIII site (step 4); Ap^r; 6.4 kb	This work
pJFF350	ColE1 replicon containing transcriptional terminators of the Ω-Km transposable element; Km^r; 5.3 kb	15
pJB17	Derivative of pUC19 in which the *Xba*I site in the polylinker was filled in (step 1) and the polylinker *Pst*I site was converted to *Xba*I (step 2); Ap^r; 2.7 kb	This work
pJB1725	The 3.6-kb blunt-ended *Hin*dIII fragment containing the Ω transcriptional terminators and the Km^r gene from pJFF350 was cloned into the *Hin*cII site of pJB17 (step 1), and the Km and *ori* region (3.0 kb) from pBR322 was deleted by *Sty*I digestion (step 2); Ap^r; 3.3 kb	This work
pJB1726	The *Xba*I site in pJB1725 was converted to a *Hin*dIII site; Ap^r; 3.3 kb	This work
pJB652	Derivative of pJB651 in which the Ω transcriptional terminators of pJB1726 were cloned as a 0.6-kb *Hin*dIII/*Eco*RI fragment into pJB651 digested with the same enzymes; Ap^r; 7.0 kb	This work
pJB653	Derivative of pJB652 in which the *Pst*I fragment containing the *Pm* promoter was cloned in the opposite orientation by digesting pJB652 with *Pst*I followed by religation (this step was necessary since DNA sequencing showed that *Pm* was in the incorrect orientation in pJB652); Ap^r; 7.0 kb	This work
pJB654	The *Xba*I site upstream of *parDE* in pJB139 and the *Bbs*I site upstream of *xylS839* in pJB653 were filled in (step 1) (originally, there were two *Xba*I sites and two *Bbs*I sites flanking *parDE* and *xylS839*, respectively), and the 3.0-kb *Sfi*I/*Bbs*I (*Bbs*I made blunt) fragment of pJB653 was replaced with the 3.8-kb *Sfi*I/*Xba*I (*Xba*I made blunt) *parDE*-containing fragment from pJB139; Ap^r; 7.8 kb	This work
pTS174	pACYC184 replicon; carries *xylR*; Cm^r	34
pJB101	Derivative of pUC7 in which a 2.4-kb *xylR*-containing *Hpa*I fragment was cloned into the polylinker *Hin*cII site of pUC7; Ap^r; 5.1 kb	This work
pJB13	Derivative of pJB8 in which the *xylR* gene of pJB101 was cloned as a 2.4-kb *Bam*HI fragment into the *Bgl*II site of pJB8; Ap^r; 6.9 kb	This work
pRD579	R1 replicon; carries the *Pu* promoter; Cb^r	11
pUC18	ColE1 replicon; Ap^r; 2.7 kb	50
pJB105	Derivative of pUC18 in which the *Pu* promoter was cloned as a 0.3-kb *Eco*RI/*Bam*HI fragment from pRD579 into pUC18 digested with the same enzymes; Ap^r; 3.0 kb	This work
pJB134	Derivative of pJB13 in which the *Pu* promoter was cloned as a 0.4-kb *Eco*RI/*Pst*I fragment from pJB105 into pJB13 digested with the same enzymes; Ap^r; 7.0 kb	This work
pJB136	Derivative of pJB134 in which the *Eco*RI site upstream of the *Pu* promoter was filled in (step 1) and the *Bam*HI site downstream of *Pu* was converted to *Eco*RI (step 2); Ap^r; 7.0 kb	This work
pJB137	Derivative of pJB136 in which the Ω transcriptional terminators from pJB1725 were cloned as a 0.6-kb *Eco*RI/*Xba*I fragment into pJB136 digested with the same enzymes; Ap^r; 7.6 kb	This work
pJB139	Derivative of pJB137 in which the *Xba*I site was filled in (step 1), the *Tth*I site was converted to *Xba*I (step 2), and the *parDE* fragment from pHL12 was inserted into the *Xba*I site as a 0.8-kb *Xba*I fragment (step 3) (the *parDE* gene is transcribed counterclockwise to the *xylR* gene); Ap^r; 8.4 kb	This work
pTB16	ColE1 replicon; Ap^r; 4.3 kb	6
pUC7*celB*	Derivative of pUC7 in which the 1.9-kb blunt-ended *Sph*I *celB* fragment from pTB16 was cloned into the *Hin*cII site of pUC7; Ap^r; 4.6 kb	This work
pJB137*celB*	Derivative of pJB137 in which the 1.9-kb *Bam*HI *celB* fragment from pUC7*celB* was cloned into pJB137 digested with the same enzyme (*celB* is transcribed from the *Pu* promoter); Ap^r; 9.5 kb	This work
pJB653*celB*	Derivative of pJB653 in which the 1.9-kb *Bam*HI *celB* fragment from pUC7*celB* was cloned in pJB653 digested with the same enzyme (*celB* is transcribed from the *Pm* promoter); Ap^r; 8.9 kb	This work

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Cb^r, carbenicillin resistance.

and induced cells increase strongly, although the induced cells express much more of the enzyme.

Figure 4B shows the results of a corresponding expression study of pJB653*celB*, containing the *Pm* promoter. The results demonstrate that the leakage expression of this promoter is not growth phase dependent and that the background level of expression is much higher than that in exponentially growing cells containing pJB137*celB* (see the legend to Fig. 4). Stimulation of the *Pm* promoter resulted in levels of expression of CelB much higher than those from *Pu*. The levels of phosphoglucomutase dropped significantly at prolonged incubation times, in contrast to what was observed in the experiments with the *Pu* promoter. Similar kinetics of induction from *Pm* have been previously reported (43).

Use of pJB653*celB* for studies of effects of *celB* expression on amylose accumulation in *E. coli*. Amylose accumulation in *E.*

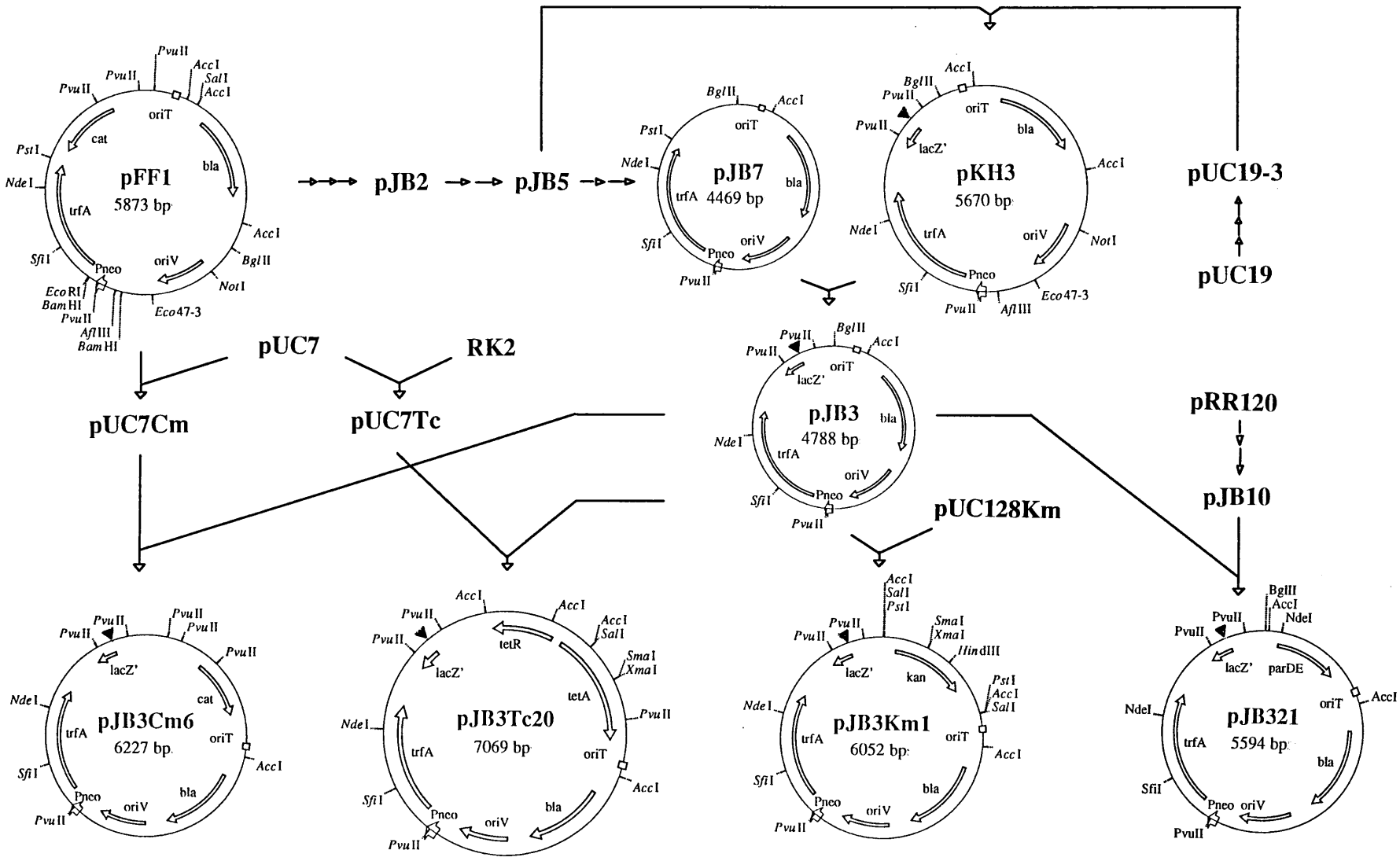

FIG. 1. Map and construction of general-purpose broad-host-range cloning vectors. Restriction sites relevant for the construction or use of the vectors are shown. Each step in the construction is indicated by an arrow. The restriction sites in the polylinker downstream of the *lacZ* promoter are marked (▼), and the sites are, in the counterclockwise direction, *Hin*dIII, *Sph*I, *Pst*I, *Sal*I/*Hinc*II/*Acc*I, *Xba*I, *Bam*HI, *Xma*I/*Sma*I, *Kpn*I, *Sac*I, and *Eco*RI. Sites in the polylinker that are not unique are indicated elsewhere on each vector. Note that the sites for *Nde*I and *Sfi*I are unique for all of the vectors except pJB321. *Pneo*, promoter from the neomycin resistance gene; *bla*, *kan*, *tet*, and *cat*, genes encoding ampicillin, kanamycin, tetracycline, and chloramphenicol resistance, respectively. The Cm, Km, Tc, and *parDE* markers were also cloned in the opposite orientations relative to the orientations in pJB3Cm6, pJB3Tc20, pJB3Km1, and pJB321. The corresponding constructs were designated pJB3Cm10, pJB3Tc19, pJB3Km2, and pJB313, respectively. See Table 1 for further details regarding the cloning steps.

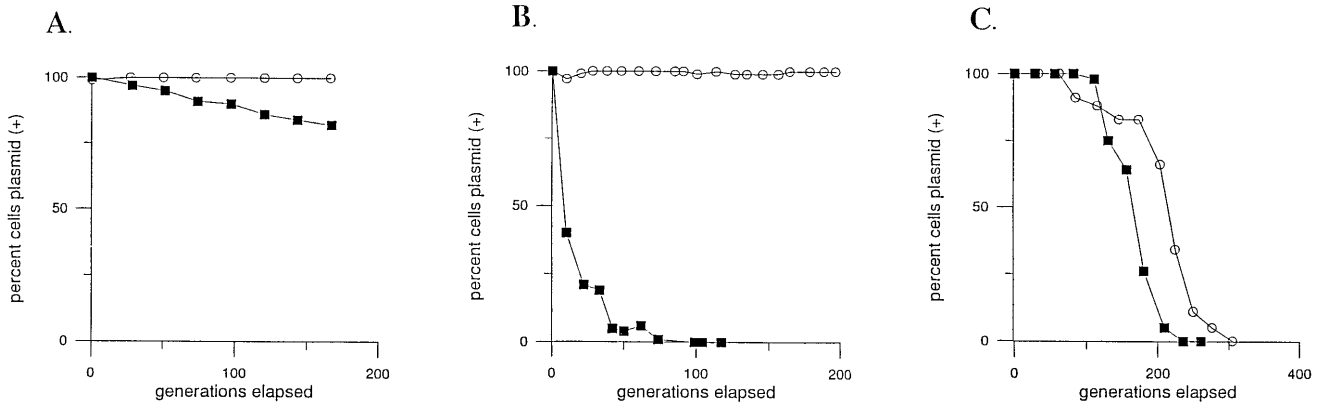

FIG. 2. Broad-host-range stabilization properties of the 0.8-kb *parDE* region in vector pJB321E. (A) *E. coli* DH5α; (B) *A. vinelandii*; (C) *P. aeruginosa*. Symbols: ■, pJB3*E*; ○, pJB321*E*. Cell cultures growing under selective conditions were diluted (at zero generations) and were then grown further under nonselective conditions. To achieve the required number of generations, the cultures were diluted several times. Plasmid-containing cells were identified by their ampicillin resistance and by their formation of yellow colonies on agar medium sprayed with a cathecol solution. For further details, see Materials and Methods.

coli was used as a model to study the intracellular effects of varying CelB expression from *Pm*, since *E. coli* cells lacking phosphoglucomutase (in contrast to the wild type) accumulate amylose intracellularly when grown on maltose as a carbon source (3). Figure 5 demonstrates that when cells are grown on maltose, amylose accumulates in similar quantities as cellular protein in PGM1. In the presence of a low level of expression of *celB* (uninduced state of *Pm*), amylose accumulation is only slightly affected. In other words, the leakage synthesis is not sufficiently high to block amylose accumulation, illustrating that this promoter system can be used to analyze rate-limiting steps in metabolic pathways. In the presence of the inducer, amylose accumulation is strongly reduced, as expected, in response to the increase in the intracellular phosphoglucomutase level. However, we found it surprising that a significant accumulation still takes place in spite of the presence of very high levels of phosphoglucomutase. We believe that this effect is somehow the result of the particular biochemical properties of the *A. xylinum* phosphoglucomutase enzyme. This is clearly illustrated by the observation that the phosphoglucomutase-positive parent strain of PGM1, Hfr3000, does not accumulate measurable quantities of amylose even in the absence of CelB (6). The activity level of the *E. coli* phosphoglucomutase in Hfr3000 is as low as about 2% of the induced-state CelB activity (data not shown). This test system therefore seems to illustrate a case where a metabolic process can be modified by replacing an enzyme in a given host by a heterologous variant.

Vector pJB653 can also be used for tightly regulated expression in *X. campestris*. To make sure that the *Pm*-based expression system is also useful in other bacteria, we transferred pJB653*celB* to a *xanA*-negative mutant of *X. campestris* and then analyzed *celB* expression in this host. *xanA* has previously been shown to encode a bifunctional phosphoglucomutase-phosphomannomutase (40), while we have found that CelB is specific for glucose-1-phosphate or glucose-6-phosphate (6). As expected, the *xanA* mutant containing pJB653*celB* did not produce detectable levels of xanthan (data not shown), while CelB was expressed at very high levels under induced conditions (Fig. 6). The expression level under uninduced conditions was about 250-fold lower, demonstrating very tight control.

DISCUSSION

The RK2-based replicons described here are small and well characterized with respect to both DNA sequences and functional properties. Due to the enormous diversity of microorganisms, many practical problems may still occur. These are, in our experience, most commonly related to inconvenient selectable markers, plasmid instability, or problems with transfer frequencies. All of these potential problems have at least been strongly reduced by the incorporation of four different selectable markers, the *parDE* sequences for stabilization of plasmid maintenance, and *oriT* to allow mobilized conjugative transfer. We have transferred these vectors to many different bacterial species, and the only organism with which we have experienced problems is *A. xylinum*. The vectors described in this report are derived from pFF1, and this plasmid can be transferred to and stably maintained in *A. xylinum*, but for some reason the expression vectors carrying the *celB* gene, pJB137*celB* and pJB653*celB*, could not be established (unpublished data). We believe that this problem is related to the sensitivity of the organism to even minor elevations in plasmid copy number (29), and we are currently investigating the possibility that this type of problem may be solved by introducing certain mutations in the *trfA* gene. These observations therefore illustrate the advantage of using replicons with a well-known biology for development of broad-host-range vectors. The vectors described here could also be easily modified and used for the studies of plasmid transfer and dispersal in natural environments.

In addition to their use as general cloning vectors, we were interested in developing the RK2-derived plasmids as tools for controlled enzyme expression in many species. Such vectors can also be used to perform pathway and other types of analyses at intermediary or very low expression levels. We believe that the vectors reported here have several advantages for such studies. In addition to the broad host range of the vectors, the *Pu* and *Pm* promoters give a very high induced-to-uninduced ratio. The maximum expression level seems to be quite high for *Pm*, and we believe that this promoter is more useful than *Pu* for most purposes. The main disadvantage with *Pu* in expression studies is its complex regulation. Expression from *Pu* has been shown to be dependent on the σ^{54} factor (7, 11, 39, 56), integration host factor (2, 10), and the growth phase (32). The

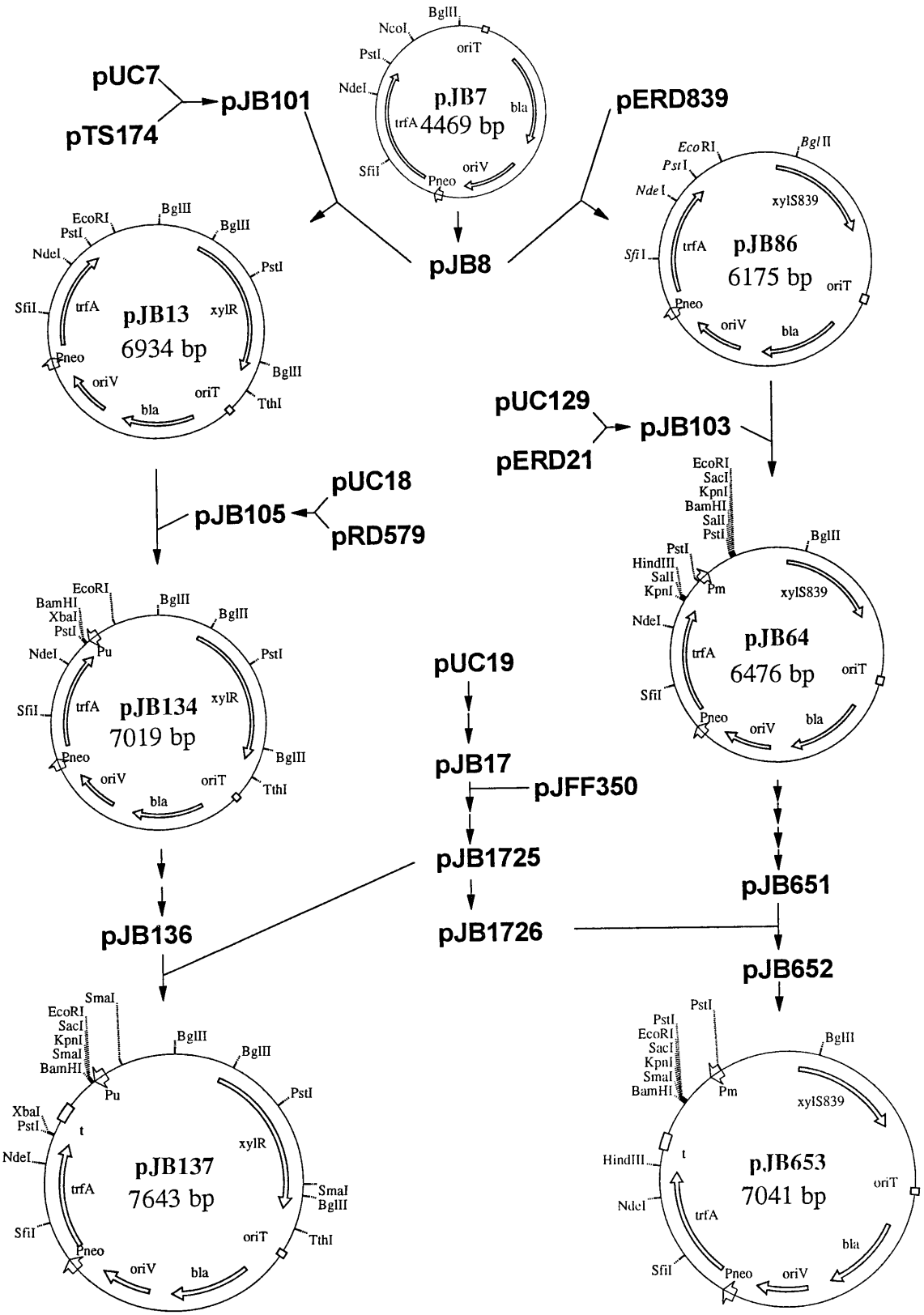

FIG. 3. Map and construction of broad-host-range expression vectors pJB137 and pJB653. The sites in the polylinker (originally from pUC19) downstream of the promoters *Pm* and *Pu* are indicated. Other notations are as described in the legend to Fig. 1. *Nde*I and *Sfi*I are unique in all of the vectors except the *parDE* derivatives pJB139 and pJB654 (Table 1). All cloning steps are described in Table 1.

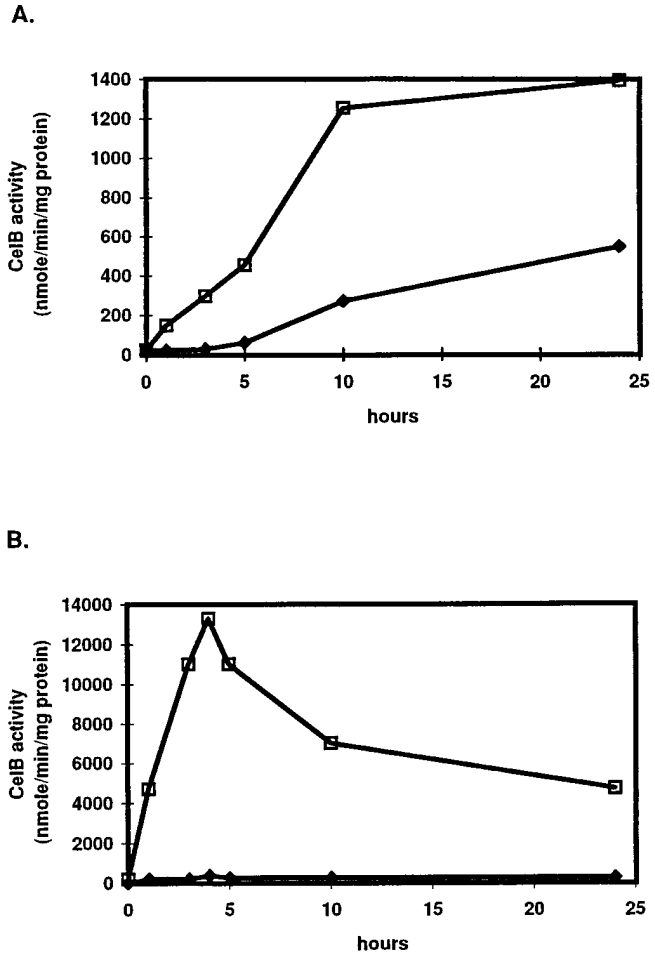

FIG. 4. Expression of *celB* as a function of cell growth for *E. coli* PGM1. (A) Expression from pJB137*celB* (*Pu*). (B) Expression from pJB653*celB* (*Pm*). Cells were grown overnight in selective medium, diluted 100-fold in the same medium, and then grown exponentially to an OD_{660} of 0.1. The inducer *m*-toluic acid was then added at 2 mM for cells containing pJB137*celB* (time zero). Cells containing pJB137*celB* were first diluted again 2,000-fold (to eliminate CelB background remaining from stationary phase) and grown in the same medium to an OD_{660} of 0.1. The inducer 3-methylbenzylalcohol was then added at 3 mM (time zero). Aliquots were removed at the times indicated to measure CelB activity in crude cell extracts. The basal level of expression of *celB* from *Pm* is between 200 and 300 nmol/min/mg of protein. □, presence of inducer; ◆, absence of inducer.

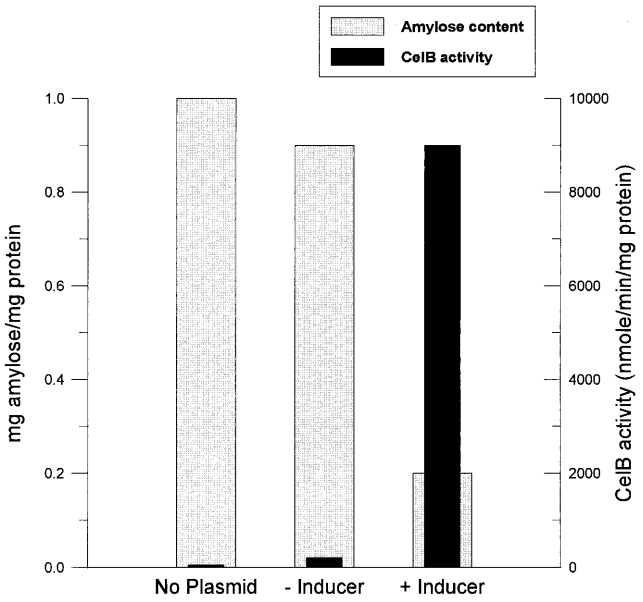

FIG. 5. Amylose accumulation in *E. coli* PGM1 as a function of *celB* expression from pJB653*celB*. Overnight cell cultures were diluted 200-fold and were then grown exponentially to an OD_{660} of 0.1. The inducer (*m*-toluic acid) was then added at 2 mM. CelB activities and amylose accumulation were determined in the same extracts 16 h after addition of the inducer.

activity of the promoter is also strongly affected by the sugars present in the growth medium (12, 30). As demonstrated in this report, the *Pu* promoter also becomes leaky in stationary-phase cells but has the advantage that leakage expression is very low in exponentially growing cells.

Expression from *Pm* does not seem to be affected significantly by the stage of growth, but leakage and maximum expression are higher than for *Pu*, even though in the constructs reported here we have used a *xylS* mutant which displays lower background expression than wild-type *xylS*. In spite of this, the amylose experiment with *E. coli* demonstrates that the background level is sufficiently low to allow control of the pathway leading to polysaccharide accumulation. It remains to be seen if other pathways require lower leakage synthesis to block or strongly reduce the flow of metabolites, but it should be possible to solve such problems by isolating expression-down mutants of the *Pm* promoter. Such variants have been described (37, 38).

The properties of the *Pm* promoter in *X. campestris* have been studied previously, and a rather low induced-to-uninduced ratio was obtained (46). Our data show that it is possible to obtain a 250-fold induction level with this promoter in *X. campestris*. The maximum level of expression in this host is high, based on the observation that CelB can be visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as one of the quantitatively dominant proteins.

Transcription from the *Pu* and *Pm* promoters can be activated by different inducers, and it is interesting that these compounds also lead to different levels of promoter activation (1, 58). This property can obviously be used to fine-tune expression levels, and in combination with the possibility of changing plasmid copy numbers by genetic means, it should be possible to regulate expression levels over a wide range. We are in the process of studying this possibility in more detail in our laboratory, and an even wider applicability, obtained by performing a mutant screening of the promoters, can be envi-

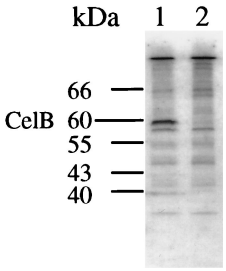

FIG. 6. SDS-PAGE analysis of the expression of CelB in *X. campestris* carrying pJB653*celB*. The inducer, *m*-toluic acid (2 mM), was added to exponentially growing cells. The cultures were then incubated further for 24 h, followed by harvesting, sonication, centrifugation, and separation of the soluble fraction by SDS–8% PAGE as described by Laemmli (41). Lane 1, induced state; lane 2, uninduced state. The levels of expression of CelB from *Pm* in the absence and presence of inducer are 800 and 206,000 nmol/min/mg of protein, respectively.

sioned. In conclusion, we believe that the vectors described here have a broad application potential. Since they are well understood physically and functionally, further modifications and improved derivatives can easily be constructed.

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