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GENETIC TRANSFORMATION AND HYBRIDIZATION

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A non-antibiotic marker for amplification of plant transformation vectors in *E. coli*

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Abstract Concern over perceived risks from the presence of antibiotic resistance genes in transgenic plants is leading to selection strategies which do not use antibiotic resistance genes as selectable markers. The concern regarding the presence of antibiotic resistance genes has focused particularly on the *bla* gene for ampicillin resistance, which is used as a bacterial marker when pUC-based plasmids are amplified prior to microprojectile bombardment. The *rtl* operon, which permits the use of ribitol as the sole carbon source, is present in *Escherichia coli* strain C but absent in all the K-12 laboratory strains commonly used for molecular biology. A *Cla*I fragment containing the kinase, dehydrogenase and transporter components of the *rtl* operon was isolated from strain C and subsequently used to replace *bla* from two cloning vectors, pBluescript and pMECA, to create pBluescript-R and pMECA-R. *E. coli* K-12 strain DH10B transformed with either plasmid acquired the ability to grow on ribitol as the sole carbon source, as long as pMECA-R was present within the bacterial cell. Plasmid yields were evaluated and found to be comparable to those obtained from bacteria growing on LB medium supplemented with ampicillin. Hence, the use of genes from the *rtl* operon might be an acceptable alternative to the use of *bla*.

Keywords Antibiotic resistance · *E. coli* strain C · Positive selection · Ribitol

Abbreviations *ACNFP* Advisory Committee on Novel Foods and Processes · *bla Beta-lactamase* gene · *LB* Luria-Bertani · *MCS* Multiple cloning site · *NCTC* National Collection of Type Cultures · *rbt* Ribitol operon from *Klebsiella pneumoniae* · *rtl* Ribitol operon from *Escherichia* coli · *TB*: Terrific Broth

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Introduction

The advent of microprojectile bombardment (Klein et al. 1987) made it possible to insert entire plasmids into plant cells. Since pUC- (Viera and Messing 1982) based vectors are frequently used for plant transformation, the resulting plants will usually contain the β-lactamase gene (known as *bla* or Ap^R), which confers resistance to the antibiotic, ampicillin, and which is used to ensure maintenance of pUC-based plasmids containing cloned genes within laboratory strains of *Escherichia coli*. The *bla* gene was originally isolated from *Salmonella paratyphi* B, in London, in 1963 (Datta and Kontamichalou 1965).

The US Food and Drug Administration has determined that there is no significant risk to the use of certain genes for antibiotic resistance in plants (FDA 1998). Nevertheless, the presence of the *bla* gene in transgenic plants has made their regulatory approval difficult after the United Kingdom's ACNFP expressed a concern that *bla* could be transferred from transgenic plants to pathogenic bacteria, thereby rendering ineffective medical treatments with ampicillin and its derivatives. The ACNFP has followed up on its original concern with a call for the elimination of all antibiotic resistance genes in transgenic plants (Tomlinson 1999). The European Union Scientific Steering Committee (SSC 1999) has also issued a similar call. Under the current regulatory environment, alternatives to antibiotic selection are desirable, although ultimately it will be up to the various regulatory agencies to determine if there are any legitimate concerns associated with the use of alternative selection strategies.

Thus far, repression-titration is the only published available alternative to the presence of an antibiotic resistance gene on a plasmid (Williams et al. 1998). This method requires engineering the bacterial chromosome with an antibiotic resistance gene behind the *lac*Z promoter, with additional binding sites for the repressor placed on the plasmid itself. Another obvious but labor-intensive way to eliminate *bla* from plasmids prior to mi-

croprojectile-mediated transformation is the enzymatic removal of *bla* from plasmids, followed by purification of the resulting fragment away from the remainder of the vector. An alternative strategy could be based on a gene that provides *E. coli* with the ability to use a carbon or nitrogen source that it ordinarily is unable to metabolize. Such potential carbon sources include ribitol and D-arabitol, which are the two most common five-carbon polyols found in nature. Strains of *Aerobacter*, *Azotobacter*, *Klebsiella* and *Pseudomonas* can utilize ribitol and arabitol as carbon sources (Reiner 1975).

E. coli strains B and K-12, from which the most commonly used laboratory strains are derived, cannot metabolize these pentitols. However, *E. coli* strain C (= NCTC strain 122; Bertani and Weigle 1953; Wiman et al. 1970), originally isolated in the Lister Institute, London, in 1920 (NCTC 1983) can metabolize both D-arabitol and ribitol and thus grow on these pentitols when they are the sole carbon source. This ability is due to the presence of two tightly linked operons (*atl* and *rtl*) in strain C, which are completely absent in strains K-12 and B. Furthermore, the conjugative transfer of these operons from strain C to strain K-12 or B permits these two strains to grow on D-arabitol or ribitol as the sole carbon source (Reiner 1975).

The same operons are found in *Klebsiella pneumoniae*, in which they are called *dal* and *rbt*, and which have been cloned and sequenced (Hartley 1984; Heuel et al. 1998). Based on this information, we decided to clone the *rtl* operon for ribitol metabolism from *E. coli* strain C and use *rtl* to replace *bla* in plasmids for microprojectile-mediated transformation. We chose ribitol over D-arabitol because ribitol is cheaper than D-arabitol and because the ribitol operon is about a half kilobase smaller than that for D-arabitol metabolism.

Materials and methods

Initial work was done with clones of the operon for ribitol metabolism (*Rbt*) from *Klebsiella pneumoniae*, supplied by S. Turgot, Universität Osnabrück. From these, a *Bam*HI fragment approximately 7.2 kb long was obtained from pFCK1, which contains the entire *Rbt* operon plus approximately 2.3 kb of sequences 3′ from the operon. A *Hin*dIII-*Bam*HI fragment approximately 6.51 kb long was obtained from pLTH9, which lacks the first 720 bp from the repressor. Finally, a *Cla*I fragment approximately 3.98 kb long was obtained from pLTH1, which lacks the repressor altogether as well as any sequences 3′ to the operon. All bacteria were grown at 37°C and shaken at 275–300 rpm.

The *K. pneumoniae* fragments were released via enzymatic digestion as recommended by the manufacturer (NEB, Beverly, Mass.) and blunted into the *Stu*I site of pMECA (Thomson and Parrott 1998). Following T4 DNA ligation with Fastlink ligase (Epicentre, Madison, Wis.), pMECA was transformed via electroporation into *E. coli* strain DH10B (Life Technologies, Gaithersburg, Md.) and placed in 2B minimal medium as recommended by BRL (Bethesda, Md.). For DH10B, the 2B minimal medium was supplemented with 50 mg l^{-1} each of L-leucine and L-isoleucine, 1 mg l^{-1} thiamine, and 2 g l^{-1} of ribitol (= adonitol, Sigma, St. Louis, Mo.) as the carbon source. The inorganic components of the 2B medium are given in Table 1. All organic components were filter-sterilized. Only successful cloning events of *rbt* were expected to result in bacterial growth, and successful growth demonstrated that ribitol could be used to maintain a high-copy plasmid in an *E. coli* K-12 strain.

Table 1 Composition of 2B minimal medium. The final medium contains 100 ml of 2B stock I, 50 ml of 2B stock II and 20 ml of 2B stock III per liter

Stock	Component	Amount per 100 ml
2B stock I	NH_4Cl	2 g
	KH_2PO_4	6 g
	Na_2HPO_4	12 g
2B stock II	$MgSO_4 \cdot 7H_2O$	0.26 g
2B stock III	$CaCl_2 \cdot 2H_2O$	0.37 g

Next, the corresponding genes of the *rtl* operon were isolated from *E. coli* strain C, which was obtained as stock number 3121 from the *E. coli* Genetic Stock Center at Yale University, and grown in 2B minimal medium supplemented with 2 g l^{-1} ribitol. Total genomic DNA was isolated according to Syn and Swarup (2000). The genomic DNA was subject to digestion by *Cla*I. Following T4 DNA ligation into the corresponding site of pHEX3 (Heuel et al. 1997) and transformation into DH10B, incubation took place in 5 ml of 2B minimal medium supplemented as already described. After 24 h, 1 ml was placed in 25 ml of the same medium. As before, only successful cloning events could result in bacterial growth. Bacterial colonies were subsequently obtained following plating an aliquot of the liquid medium onto plates containing solid LB medium supplemented with 20 mg l^{-1} chloramphenicol. A single colony was grown in TB (Terrific Broth, Tartof and Hobbs 1987); plasmid DNA was isolated using a Quantum-Prep kit (BioRad, Hercules, Calif.) and sequenced using a Model 377 automated DNA sequencer (PE Applied Biosystems, Forster City, Calif.) by the University of Georgia Molecular Genetics Instrumentation Facility. Sequence comparisons were made using the on-line BLAST programs of the National Center for Biotechnology Information (Bethesda, Md.) and the GAP program of the Wisconsin Package, version 10.1 (Genetics Computer Group, Madison, Wis.).

To replace *bla* from pBluescript (Stratagene, La Jolla, Calif.) and pMECA with the *rtl* components, the *Cla*I fragment was liberated with enzymatic digestion, blunt-ended and isolated as a 4-kb fragment from a low-melting-point SeaPlaque agarose gel (FMC Bioproducts, Rockland, Me.). The recipient plasmids were cut open with *Ahd*I, blunted with T4 DNA polymerase and digested with *Ssp*I to remove nearly the entire sequence of the *bla* gene. The fragment was ligated into the plasmids usingT4 DNA ligase. These events are diagrammed in Fig. 1. Ligated plasmids were transformed into DH10B and grown in 2B minimal medium supplemented as described above. The resulting plasmids were named pMECA-R and pBluescript-R, respectively. Plasmid maps were drawn with Plasmid 1.1 (Redasoft, Toronto, Ont.).

Growth rates and plasmid yields were compared for pMECA, pMECA-R, pBluescript and pBluescript-R in DH10B. Both pMECA and pBluescript were grown in LB broth and in 2B minimal medium supplemented with 2 g l^{-1} glucose and L-leucine, L-isoleucine and thiamine as described above. pMECA-R and pBluescript-R were grown in 2B minimal medium supplemented likewise, except that ribitol was substituted for glucose. Bacteria were inoculated into 2 ml of their respective media, allowed to grow for 17 h or 41 h and plasmid DNA isolated as described previously. Plasmid yield was quantified with a DNA fluorometer (Hoefer Scientific, San Francisco, Calif.). Each treatment was repeated three times.

Plasmid yields were also quantified for pMECA-R and pBluescript-R containing an insert in their MCSs (multiple cloning sites). A *Pst*I-*Asc*I fragment from pTFG containing the cauliflower mosaic virus (CaMV) 35S promoter-GUS (β-glucuronidase)-nos terminator construct out of pTRA140 (Zheng et al. 1991) was ligated into the *Pst*I and *Asc*I sites of pMECA-R, and a *Pst*I-*Stu*I fragment of pTFG was ligated into the *Pst*I and *Eco*RV sites of

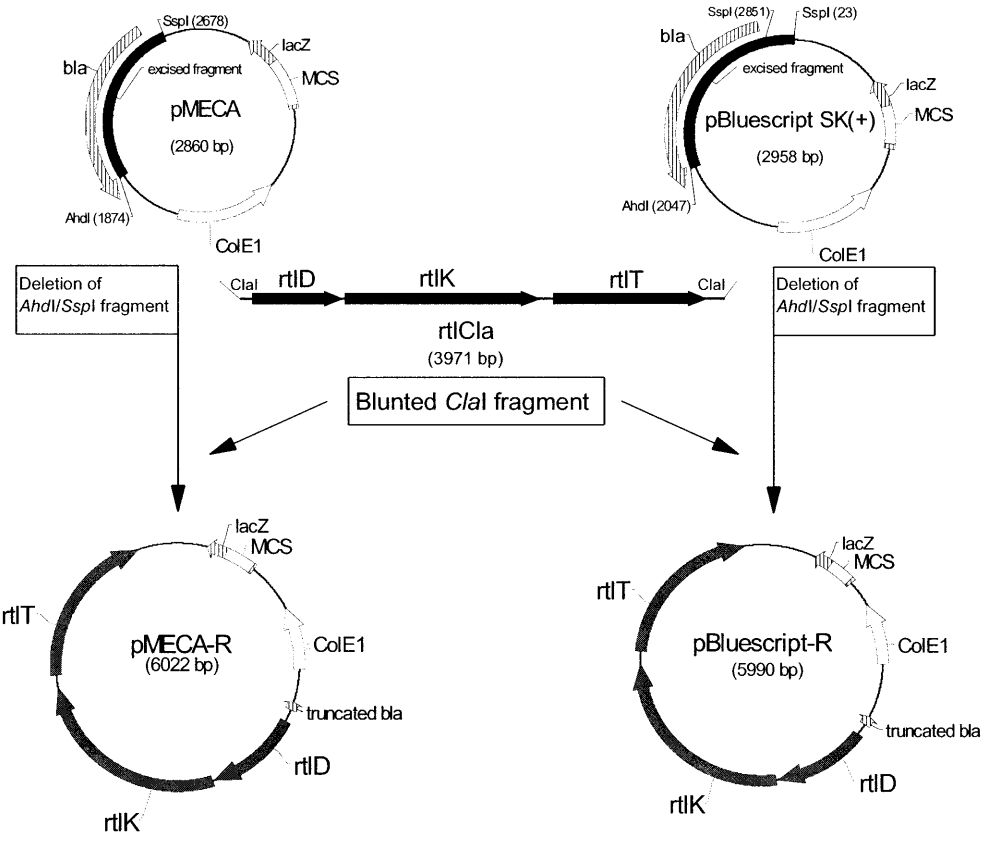

Fig. 1 Replacement of *bla* with *rtlK*, *rtlD* and *rtlT* in pMECA and pBluescript

pBluescript-R. The ligated plasmids were transformed into DH10B, cultured, isolated and quantified as described above. Plasmid yields were subjected to an analysis of variance (SAS, SAS Institute, Cary, N.C.). Since plasmid effects were highly significant, yield means were further subjected to means separation using the Least Significant Difference. As transformations on the data to reduce the heterogeneity of variance did not alter the results, raw data were used for the analyses.

Results and discussion

The ribitol operon from *E. coli* C contains four genes in the following order: *rtlR*, *rtlD*, *rtlK* and *rtlT*. The first is a repressor and contains the promoter for the operon. *RtlD* codes for NAD-dependent ribose dehydrogenase, which converts ribose to D-ribulose. *RtlK* codes for ATP-dependent ribulose kinase that converts D-ribulose to D-ribulose-5-phosphate, of which the latter can be metabolized by all *E. coli* strains. Finally, *rtlT* codes for a ribose-specific ion symporter protein (Heuel et al. 1998).

The use of the fragments from *K. pneumoniae* permitted the characterization of growth on ribitol and the definition of the operon components necessary to achieve this growth. This information then facilitated cloning of the operon from *E. coli* C. Though the *Bam*HI, *Cla*I and *Hind*III-*Bam*HI *rbt* fragments all conferred the ability to grow on ribitol, the *Cla*I fragment, which completely lacks the repressor, was all that was required for growth on ribitol; consequently, all subsequent work did not attempt to include *rtlR* in the cloning procedure. The sequence of the *rtl Cla*I fragment has been deposited in GenBank as accession AY005817. The ribitol operons of *Klebsiella* and *Escherichia* are thought to have a common evolutionary origin, and Heuel et al. (1998) reported 82% base identity between *rbt* and *rtl* based on partial sequencing. A BLAST comparison using complete sequences between the *D*, *K*, and *T* components of *rbt* and *rtl*, while revealing highly conserved regions, found greater homology at the amino acid level than the DNA level. The base pair identity for the *rbt* and *rtl Cla*I fragments was 70%. Amino acid identity was 87%, 70% and 83% between the *rbt* and *rtl* dehydrogenase, kinase and transporter components, respectively. When substitution with similar amino acids is considered, the homologies rise to 94%, 79%, and 89%, respectively.

The *bla* gene is only 788 bp long, in contrast to *rtlD*, *rtlK* and *rtlT*, which are collectively 3,971 bp long. We were able to compensate to some extent for the size difference by eliminating some of the DNA flanking the *bla* gene. Nevertheless, pMECA-R is 3,183 bp larger than pMECA, and pBluescript-R is 3,032 bp larger than pBluescript. Replacing *bla* with *rtlD*, *rtlK* and *rtlT* inevitably brought along enzyme sites which led to the loss of uniqueness of enzyme sites within the pMECA and pBluescript MCSs. Nevertheless, pMECA-R still has 30 unique sites, while pBluescript-R has 18 unique sites. These are listed in Table 2.

Plasmid yields for DH10B are depicted in Fig. 2. Heuel et al. (1998) reported that high-copy number

Table 2 Unique enzyme sites in the multiple cloning sites of pMECA-R and pBluescript-R

pMECA-R		pBluescript-R
*Eco*RI	*Nae*I	*Sac*I
*Eco*RV	*Fse*I	*Not*I
*Spe*I	*Avr*II	*Eag*I
*Not*I	*Hpa*I	*Xba*I
*Acc*651	*Hin*dIII	*Spe*I
*Kpn*I		*Sma*I
*Xho*I		*Pst*I
*Bss*HII		*Eco*RI
*Asc*I		*Eco*RV
*Xba*I		*Hin*dIII
*Sfi*I		*Hinc*II
*Bsp*120I		*Acc*I
*Apa*I		*Sal*I
*Pac*I		*Xho*I
*Pme*I		*Apa*I
*Sal*I		*Eco*O1091
*Acc*I		*Dra*II
*Xma*I		*Kpn*I
*Sma*I		
*Srf*I		
*Pst*I		
*Sph*I		
*Swa*I		
*Nhe*I		
*Ngo*MIV		

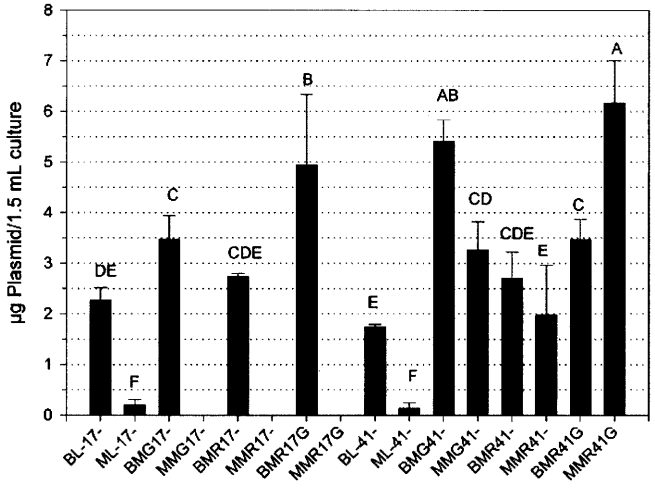

Fig. 2 Comparative plasmid yields in DH10B of pBluescript and pMECA, growing in LB broth or 2B minimal medium supplemented with glucose, and of their ribitol derivatives growing on 2B minimal medium with ribitol. Plasmid yield data were collected at 17 h and 41 h. Plasmid yields for pBluescript-R and pMECA-R with a GUS construct cloned into their multiple cloning sites were also compared. *Bars* represent the average of three replications ± standard error. Treatments identified with the *same letter* are not different from each other as determined by a protected LSD test, α=0.05. The key to the plasmids listed under the graph is as follows. The *first letter* signifies the plasmid backbone: *B* pBluescript, *M* pMECA. The *second letter* indicates the medium: *L* Luria-Bertani broth, *M* 2B minimal medium. The *third position* denotes the carbohydrate source: - no additional carbohydrate, *G* glucose, *R* ribitol. The *number* represents the allotted growth period: 17 h or 41 h. The *final position* denotes if the plasmid had an insert cloned into its multiple cloning site: – no insert, *G* a GUS construct

plasmids containing *dal* or *rbt* were unstable under arabitol or ribitol selection, but we did not experience any such difficulties.

For the Bluescript plasmids, yields were higher in 2B minimal medium than in the standard LB broth. Yields of the ribitol version were essentially like those of the ampicillin version after 17 h when grown in minimal medium. By 48 h yields of the ribitol version were lower than those of the ampicillin version. On a molar basis, yields in 2B minimal medium with ribitol are lower than those in 2B minimal medium with glucose, as the ribitol version of pBluescript is twice as large as the ampicillin version. Insertion of a GUS construct into the MCS significantly increased plasmid yield at 17 h, but this difference was not evident by 41 h.

For the MECA plasmids, yields were negligible at the 17-h time point. When pMECA lacks an insert in its MCS, the growth rate of its host cells is slowed down, an effect that is particularly pronounced in strain DH10B. This slow-growth trait is useful for identifying colonies lacking inserts (Thomson and Parrott 1998). By 41 h, growth of pMECA in 2B minimal medium was significantly greater than that in LB, although the ampicillin version outgrew the ribitol version. Yields for pMECA-R containing an insert were higher after 41 h than those for pBluescript-R after 17 h.

In principle, ribitol selection should work with any K-12 strain of *E. coli*. The K-12 strains in use today have an absolute requirement for essential nutrients in the growth medium, as the parent strain has been subject to extensive mutagenesis since its isolation in 1922 (Bachmann 1972). Since different K-12 strains have different growth requirements, the minimal growth medium must be adjusted for each K-12 strain by adding the necessary nutrients that each particular strain requires.

Reiner (1975) listed individual bacterial strains which have been identified based on their ability to metabolize either sucrose or raffinose. The cloning of these operons should permit the development of additional vectors, which would permit the use of these carbohydrates in a selection system, either for the maintenance of plasmids in bacteria or for the selection and recovery of transgenic plants. Already, vectors are available which permit the use of xylose (Haldrup et al. 1998) or mannose (Joersbo et al. 1998; Negrotto et al. 2000).

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