

Strategy based on plasmid incompatibility for Tn5 mutagenesis in *Erwinia herbicola* ATCC 21998

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Erwinia herbicola and *Gluconobacter oxydans* are ketogenic bacteria capable of oxidation of hexose sugars to a number of keto-derivatives mediated by a consortium of enzymes known as dehydrogenases. In our studies with *G. oxydans*, Tn5 transposition gave rise to block mutants in the direct glucose oxidation pathway. Some of these mutants have commercial value. But similar mutagenesis was not successful in *E. herbicola* because pSUP2021, the Tn5 carrying vector, stably replicated in this organism when transformed with the vector. Here we report a strategy for transposon mutagenesis in *E. herbicola* based on plasmid incompatibility to convert the stably maintaining plasmid pSUP2021 into a suicidal vector in presence of another plasmid pBR322::Tn5 incompatible with it. The resultant transposon generated auxotrophic mutants (for arginine and lysine) in this commercially valuable organism. The study also confirmed that the native plasmid (pVQ1) harboured by *E. herbicola* has its origin non-homologous to that of pMB1.

ERWINIA HERBICOLA (ATCC 21988), a gram-negative facultative anaerobe, is a commercially important ketogenic bacteria. It has the ability to convert glucose to 2,5-diketo-gluconic acid (2,5-DKG) and sorbitol to 2-keto-L-gulononic acid (2-KLG), intermediates of vitamin C synthesis¹. The gene encoding glucose dehydrogenase (GDH) in *E. herbicola* has been located on one of its two plasmids, pVQ1 (ref. 2). The strain carries another plasmid (pVQ2) which seems to be cryptic and was found to be curable with mitomycin C (ref. 2). However, pVQ1 could not be cured even after repeated attempts using not only mitomycin C but other mutagenic agents like acridine orange, NTG, ethidium bromide and UV radiations. Genetic analysis of specific genes and the information available on the basic genetics of *E. herbicola* is scanty. Methods of conventional mutagenesis can be used to generate mutants but preferential mutants are usually difficult to obtain. Transposons are drug-resistant elements that have been utilized successfully in recent years for the development of mutants^{3,9}. Transposon Tn5 is frequently used as a mutagen because it is a single site mutagen, does not confer any phenotypic effect on cell growth, inserts randomly, causes polar mutations and is highly transposable and stable once established^{3,4,8,9}.

A strain of *E. herbicola* (EhC1) was obtained by curing *E. herbicola* ATCC 21988 with mitomycin C (ref. 2). Here we report Tn5 transposonesis in EhC1 for generating block mutants using the phenomenon of plasmid incompatibility. Besides obtaining random transposition-yielding auxotrophs, the results reveal that native plasmid pVQ1 does not belong to Inc P group of plasmids as its origin is different from that of pMB1, to which the pBR322 derived vectors belong.

The bacterial strains used in this study are given in Table 1. The routine growth media for the bacteria was LB¹⁰ and the minimal medium for *E. herbicola* and its strain (EhC1) was the mineral salt medium¹¹. Glucose/sorbitol was used as carbon source at a concentration of 20 g l⁻¹. For the selection of Tn5 expression, kanamycin (Km) was used at a final concentration of 50 µg ml⁻¹. Ampicillin (Am), erythromycin (Er) and chloramphenicol (Ch) were used at a concentration of 100 µg, 60 µg and 25 µg ml⁻¹, respectively.

Transposon mutagenesis in EhT1 (EhC1 bearing pBR322::Tn5) was performed by bacterial mating between EhT1 and *E. coli* S17-1 carrying pSUP2021 by following the protocol of Simon *et al.*⁹. Counter selection of donor cells was made on Er 60 µg ml⁻¹ whereas recipient cells were selected on Km 50 µg ml⁻¹ and Ch 25 µg ml⁻¹. After 24 h of incubation at 30°C, the colonies were replica plated on LB with Km 50 µg ml⁻¹ and Ch 25 µg ml⁻¹.

Total DNA was isolated from all the isolates of *E. herbicola* by following the method of Pitcher *et al.*¹². Plasmid DNA was isolated by the alkali lysis method of Birnboim and Dolly¹³. DNA was analysed by agarose gel electrophoresis using TAE buffer¹⁰. Transformation of the strains of *E. herbicola* (EhC1, EhT1) with pBR322::Tn5 (obtained from IMT, Chandigarh) and pSUP2021 (obtained from Prof. R. Simon, University of Bielfeld, Germany) was undertaken following the method of Hamamoto and Murooka¹⁴ with some modifications, i.e. (i) 100 mM KCl gave better results than 100 mM RbCl₂, (ii) PEG 1000 was found to be more effective than PEG 4000. Transformants were selected on LB medium containing specific antibiotics, viz. EhC1 pBR322::Tn5 (EhT1) on Am 100 µg ml⁻¹ and Km 50 µg ml⁻¹; EhT1 pSUP2021 (EhT2) on Km 50 µg ml⁻¹ and Ch 25 µg ml⁻¹, respectively. The selection of auxotrophs was done by following the protocols of Davis *et al.*¹⁵. The transposed colonies were replica plated on LB with Km 50 µg ml⁻¹ + Ch 25 µg ml⁻¹ and series of eleven diagnostic pool media¹⁵ comprising permutation and combination of amino acids (Table 2). The frequency of transfer of pSUP2021 from *E. coli* S17-1 through conjugation, in *E. herbicola* (wild type) or its cured derivative (EhC1) was of the order of 70% per final number of recipient cells. The electrophoresis data of the ex-conjugants, as shown in Figure 1, indicate that

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Table 1. Bacterial strains used in the study

Strain no.	Bacterial strains	Source	Phenotype/plasmids present	Growth media	Temp. (°C)
Eh	<i>E. herbicola</i>	ATCC, USA	Am ^r Cb ^r (pVQ1 & pVQ2)	LB/MM	28
EhC1	<i>E. herbicola</i>	RRL, Jammu	Am ^r Cb ^r Er ^r (pVQ1)	LB/MM	28
EhT1	<i>E. herbicola</i>	RRL, Jammu	Am ^r Cb ^r Km ^r Er ^r (pVQ1 & pBR322::Tn5)	LB/MM	28
EhT2	<i>E. herbicola</i>	RRL, Jammu	Am ^r Ch ^r Er ^r Km ^r (pVQ1 & pSUP 2021)	LB/MM	28
S17.1	<i>E. coli</i>	Pasteur Institute, Paris	Sm ^r	LB	37
S17.1 pSUP 2021	<i>E. coli</i>	Gifted by Prof. R. Simon	Am ^r Ch ^r Km ^r	LB	37
S7	<i>E. coli</i>	IMT, Chandigarh	Am ^r Km ^r (pBR322::Tn5)	LB	37

Cb = Carbenicillin; Ch = Chloramphenicol; Sm = Streptomycin; Km = Kanamycin; Am = Ampicillin; Tc = Tetracycline; Er = Erythromycin; LB = Luria broth; MM = Minimal medium. Temp. = Optimum temperature for growth.

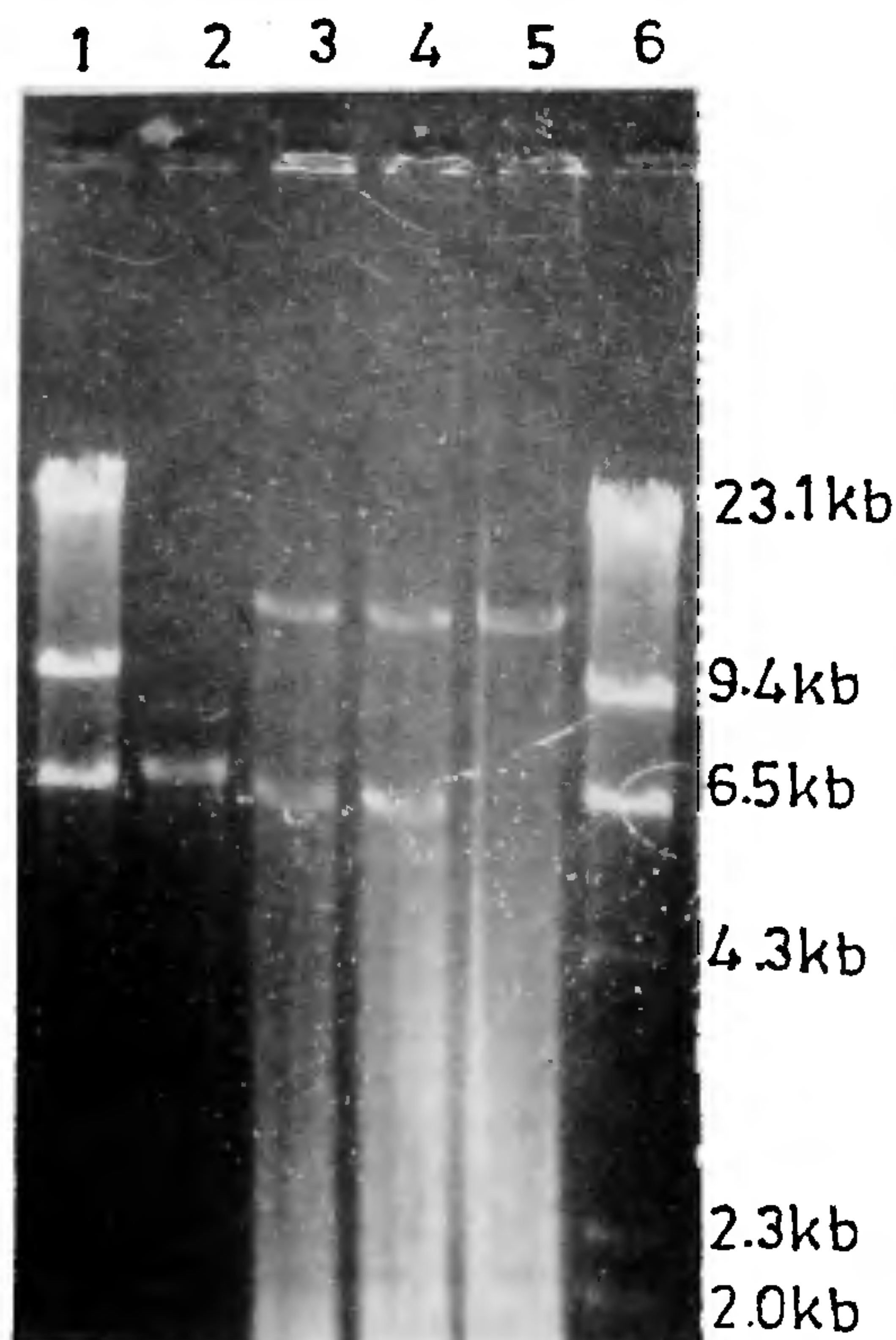


Figure 1. 0.7% agarose gel electrophoresis of plasmid DNA from *E. herbicola* (EhC1) showing presence of pSUP2021 along with pVQ1. Lane 1, λ DNA/HindIII marker; Lane 2, pVQ1/EcoRI digest; Lanes 3 and 4, EcoRI digest of the plasmid DNA preparation from EhT2 showing the presence of pVQ1 and pSUP2021. Lane 5, pSUP2021 EcoRI digest. Lane 6, λ DNA HindIII marker.

the plasmid pSUP2021 is stably maintained in EhC1, as was also found with wild type *E. herbicola* (data not shown). Thus, the plasmid pSUP2021 being non-suicidal in these strains makes it an unsuitable vector for transposon. Therefore, possibility of plasmid incompatibility was explored for affecting transposon in this organism using different vectors carrying Tn5 transposon.

The transformation frequency of pBR322::Tn5 or pSUP2021 in EhC1 was approximately of the order of 2×10^{-5} . Both of these plasmid vectors were found to stably replicate in EhC1 when transformed separately. Therefore, a strategy was designed to introduce one of these Tn5 carrying vectors into EhC1 host possessing already the other Tn5-based vector having the same origin of replication, e.g. transform EhT1 (EhC1 bearing pBR322::Tn5) with pSUP2021. As both pBR322::Tn5 and pSUP2021 have the same pMB1 origin, the two plasmids became incompatible with each other and resulted in the elimination of one, in this case pBR322::Tn5. Such eliminations triggered random transposition of Tn5 on the chromosomal DNA of the host (EhC1). The transposition of Tn5 was confirmed by hybridization using DIG-labelled probe derived from Tn5 (2.7 kb *Bgl*III fragment, Figure 2). The transposition data thus generated, suggest that it was always the incoming plasmid that maintained stability and the one already present in the host became suicidal and got eliminated. Similar observations have been reported by Berg and Berg³ with respect to pJB4J1 and pJB3J1.

Screening of 5000 ex-conjugants revealed that the transposition occurred mostly on the chromosomal DNA and never on the native plasmid DNA (pVQ1) harboured by the organism. It may be due to the presence of certain hot spots on the chromosome of *E. herbicola*.

Table 2. Various auxotroph diagnostic pool media

	1	2	3	4	5
6	Adenosine	Guanosine	Cysteine	Methionine	Thiamine
7	Histidine	Leucine	Isoleucine	Lysine	Valine
8	Phenylalanine	Tyrosine	Tryptophan	Threonine	Proline
9	Glutamine	Asparagine	Uracil	Aspartic acid	Arginine
10	Thymine	Serine	Glutamic acid	DAP	Glycine
11	Pyridoxine	Nicotinic acid	Biotin	Pantothenate	Alanine

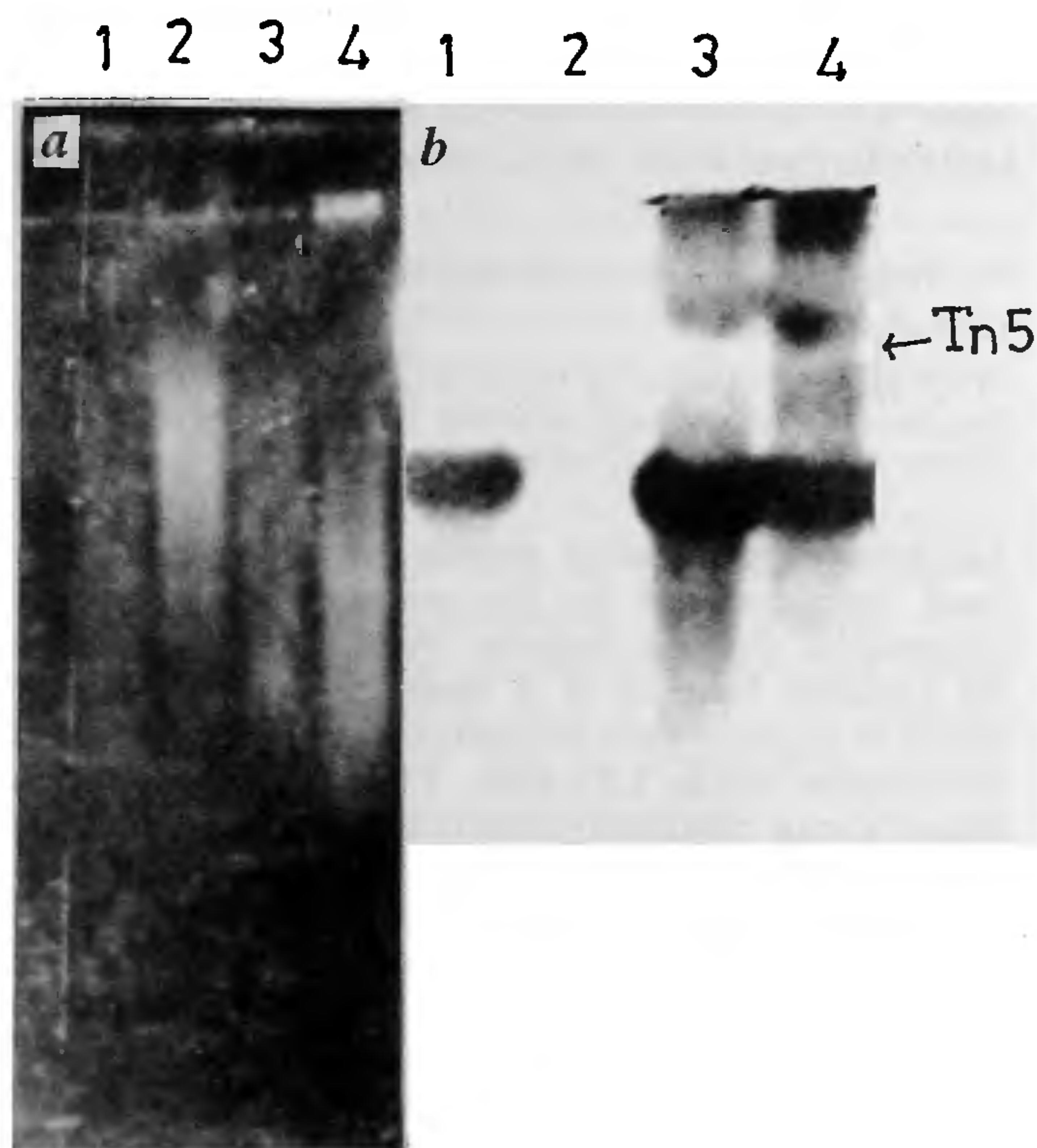


Figure 2. *a*, 0.7% agarose gel electrophoresis showing *Eco*RI digest of total DNA from: Lane 1, *E. coli* S17-1 with pSUP2021; Lane 2, *E. herbicola* (EhC1); Lane 3, Ex-conjugant Eht2 (no. 1); Lane 4, Ex-conjugant Eht2 (no. 2). *b*, Southern blot of the above gel showing hybridization using DIG-labelled *Bgl*III (2.7 kb) fragment of Tn5 as a probe. Lane 1, pSUP2021 *Eco*RI digest as the positive control; Lane 2, No hybridization with EhC1; Lanes 3 and 4, Show additional signals marked as (←) depicting the transposition of Tn5 from pBR322::Tn5 on the chromosomal DNA of Eht2. pSUP2021 *Eco*RI digest is at the expected position.

This observation is in conformity with that of Gantotti *et al.*⁶, who reported that insertion of Tn5 in *E. herbicola* was not as random as in *E. coli*. Presence of similar hot spots for Tn5 in chromosomal DNA of *Gluconobacter oxydans* has also been reported by our group earlier¹⁶.

In contrast to parent organism, some of the ex-conjugants obtained by the above-mentioned strategy failed to grow on the minimal medium with glucose as the sole carbon source. These ex-conjugants when replica-plated on 11 diagnostic pool media¹⁵ (Table 2) designed to score among others the amino acid auxotrophs, resulted in the selection of two ex-conjugants,

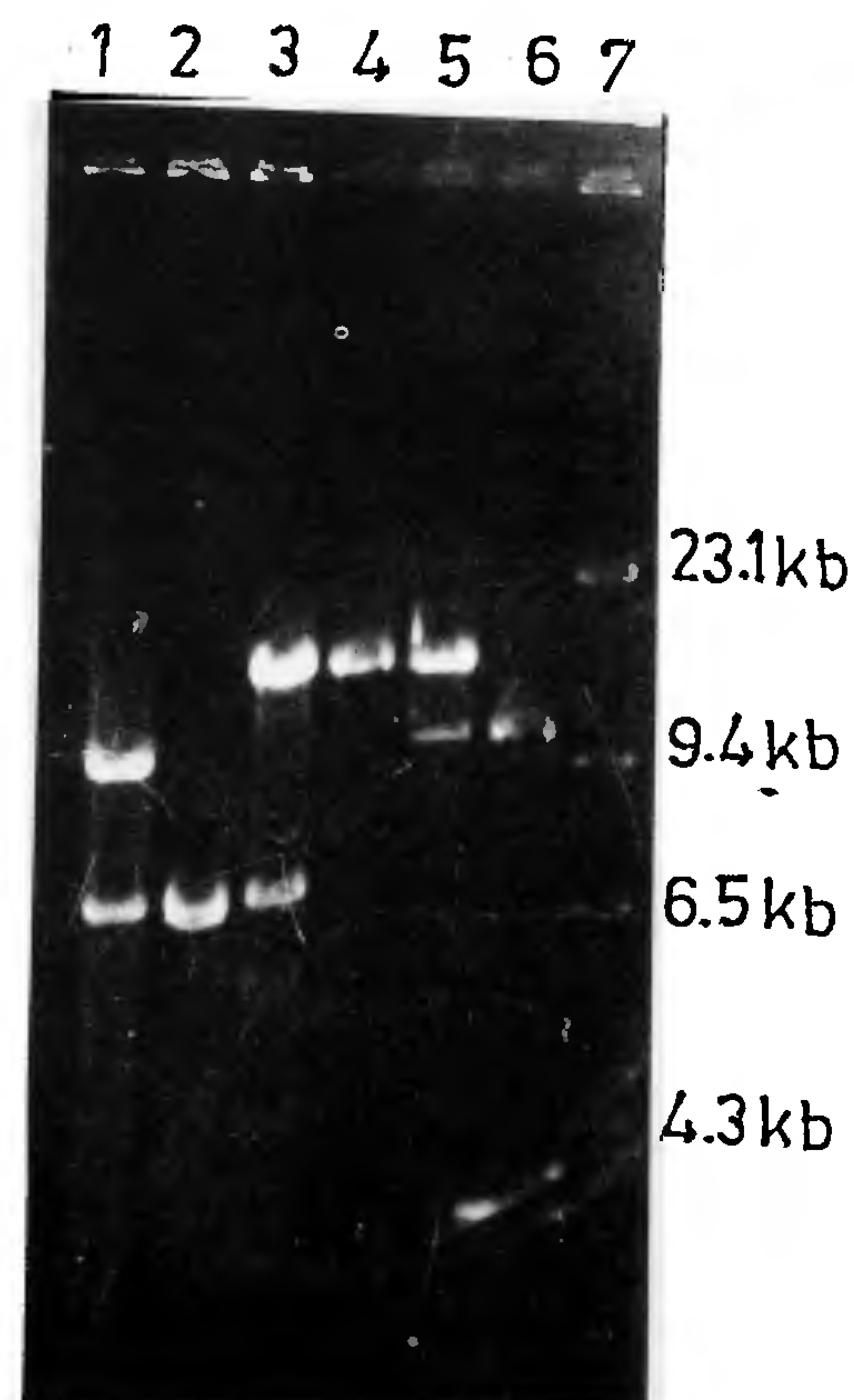


Figure 3. 0.7% agarose gel electrophoresis showing curing of pBR322::Tn5 as a result of plasmid incompatibility between pBR322::Tn5 and pSUP2021 in *E. herbicola* (EhC1) transformants. Lane 1, pVQ1 and pBR322::Tn5 from Eht1 *Eco*RI digest; Lane 2, pVQ1 of EhC1 *Eco*RI digest; Lane 3, pVQ1 and pSUP2021 from ex-conjugant Eht2 *Eco*RI digest; Lane 4, pSUP2021 *Eco*RI digest; Lane 5, Mixture of pSUP2021 and pBR322::Tn5 *Eco*RI digests as control; Lane 6, pBR322::Tn5 *Eco*RI digest; Lane 7, λ DNA *Hind*III marker.

Eh aux1 and Eh aux2. These ex-conjugants grew well on pool media 4 and 9, respectively (Table 2). When lysine was eliminated from the pool medium 4, Eh aux1 failed to grow on it. Similarly when arginine was removed from pool medium 9, Eh aux 2 could not grow. This, therefore, indicates that Eh aux1 is lysine-auxotroph and Eh aux 2 is arginine-auxotroph. Detailed studies on the mutants are under way and will be reported separately.

Attempts to transform *E. herbicola* with pBR322 (relatively smaller plasmid) alone were not successful, how-

ever pBR322::Tn5 (~10 kb) and pSUP2021 (~12 kb) were accepted easily by the culture. Therefore the preferential uptake of pBR322::Tn5 and pSUP2021 indicates that molecular weight of the plasmid alone does not determine the transformation frequency in *E. herbicola*. Similar observations have also been made in *Erwinia* sp. by other workers¹⁴. Attempts to mobilize pBR322 using *E. coli* S17-1 as the helper strain were also not successful. This is probably because of the fact that pBR322 lacks RP4 specific mob site.

Transformations of EhC1 with pBR322::Tn5 and pSUP2021 clearly show that pVQ1 (natural plasmid harboured in EhC1) has different origin of replication than that of pSUP2021 and pBR322. Both of these plasmids also co-existed stably with pVQ1 when transformed in the culture separately (Figure 3). This clearly shows that pVQ1 does not belong to Inc P group of plasmids and its origin is other than that of pMB1. During this study we did not come across any co-integrates formed as a result of homology between Tn5 present in pBR322::Tn5 and pSUP2021. This further confirms incompatibility between the two plasmids (pBR322::Tn5 and pSUP2021). Thus, it is evident that for the development of block mutants, the plasmid incompatibility strategy (using two different transposon vectors) could be exploited efficiently to facilitate transposition in *E. herbicola*.

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Agrobacterium*-mediated genetic transformation in *Gerbera hybrida

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***Agrobacterium*-mediated genetic transformation protocol was developed for the commercially important ornamental *Gerbera hybrida*. Petiole, leaf and shoot tip explants from 3 to 4 week old *in vitro* grown shoots of gerbera were co-cultured with *Agrobacterium tumefaciens* strain LBA4404. The strain harbours a binary vector containing neomycin phosphotransferase (*nptII*) and β -glucuronidase (*uidA*) genes. Callus formation and shoot regeneration were obtained on MS medium containing BAP, kinetin (1.0 mg/l each) and NAA (0.5 mg/l). Selection on the regeneration medium supplemented with 20 mg/l kanamycin allowed production of transgenic plants from 0.44% to 17.0% of the explants. GUS activity was detected by histochemical staining. Transfer of *uidA* and *nptII* genes was analysed by PCR and Southern hybridization. Transformed shoots were multiplied on MS medium containing 0.25 mg/l BAP, rooted in half strength MS medium supplemented with 40 mg/l kanamycin and successfully transferred to soil. This protocol could be used to introduce horticulturally important genes that govern pigment biosynthesis in flowers.**

ORNAMENTAL plants are produced exclusively for their aesthetic values. Thus the improvement of quality attributes such as flower colour, longevity and form, plant shape and architecture and the creation of novel variation are important economic goals¹. *Gerbera* is one of the most popular ornamental plants in the world and is used as garden plants, potted plants, greenhouse flowers and cut flowers. Conventional breeding of gerbera by crossing and selection has generated several clonally propagated elite genotypes that have desirable traits, such as colour, shape, vase life and resistance against pests and diseases. However, one of the disadvantages of traditional breeding

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