Plasmids Formed in Nitrogen-fixing *Escherichia coli*-*Klebsiella pneumoniae* Hybrids

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SUMMARY

Segments of the *Klebsiella pneumoniae* chromosome, which included the nitrogen fixation (*nif*) genes, were maintained as covalently closed circular molecules of DNA in *Escherichia coli* hybrids, C-M9 and C-L4. The R factor, R144drd3, which had been used to confer fertility on the donor, was also detected as covalently closed circular DNA of molecular weight $(65 \pm 4) \times 10^6$ daltons according to sedimentation studies and electron microscopy.

The *nif* genes in hybrid C-M9 were linked to *his* on a plasmid which had a molecular weight of 9.5×10^6 daltons. A third plasmid in this hybrid had a molecular weight of 118×10^6 daltons. The Nif plasmid in C-M9 was transferred efficiently to other *Escherichia coli* strains only when R1drd19 was present in the donor in addition to R144drd3.

Presence of three electrophoretically distinguishable gluconate-6-phosphate dehydrogenases was evidence for duplication of *gnd* determinants in C-M9. Resistance of both hybrids to the rough-specific phage ϕ X 174 suggested that they inherited *Klebsiella* *his*-linked *rfb* genes.

Four plasmids were isolated from hybrid C-L4, one of which was R144drd3. Two plasmids, one carrying *his* and *nif* and another *met* G, were mobilized by R144drd3. The molecular weights of the C-L4 plasmids, excluding R144drd3, were $(105 \pm 1) \times 10^6$, 46×10^6 and 12×10^6 daltons.

INTRODUCTION

Genetic crosses between *Escherichia coli* Hfr strains and *Salmonella typhosa* recipients generate hybrids in which the inherited *E. coli* genes have not undergone recombination with the recipient chromosome (Baron, Gemski, Johnson & Wohlhieter, 1968). A comparable phenomenon has been observed in matings between *E. coli* and *Shigella flexneri* (Falkow, Schneider, Baron & Formal, 1963), between *E. coli* and *Proteus mirabilis* (Gemski, Wohlheiter & Baron, 1967) and between *E. coli* and *Salmonella typhimurium* (Mojica-a & Middleton, 1972). In the crosses between *S. typhimurium* and *E. coli*, exogenote hybrids in which the *E. coli* DNA did not integrate into the chromosome occurred at about the same frequency as haploid hybrids.

Analysis of their DNA revealed that the *Escherichia coli* genetic segments transferred were conserved in *Salmonella typhosa* as covalently closed circular (CCC) molecules (Leavitt *et al.* 1971). Though a range of sizes was observed among the CCC molecules of the hybrids, each contained CCC molecules of a single characteristic size consistent with the presence in each of a different length of *E. coli* chromosome.

Intergeneric matings between *Klebsiella pneumoniae* and an *Escherichia coli* strain C

recipient generating His⁺ Nif⁺ *E. coli* hybrids were reported by Dixon & Postgate (1972). In a companion to this paper (Cannon, Dixon, Postgate & Primrose, 1974), we have presented data concerning a stable hybrid, strain C-M7, which led to the conclusion that the *Klebsiella nif* operon had probably become integrated into the *E. coli* chromosome as a segment of *Klebsiella* DNA carrying also *his* and the unselected markers *gnd* and *rfb*. Dixon & Postgate (1972) reported the formation of three less stable hybrids which segregated Nif⁻ segregants at high but variable frequencies. For one, strain C-M8, continued positive selection was necessary to conserve the hybrid state and it has now been lost. We now report a study of the two remaining hybrids, strains C-M9 and C-L4, including physico-chemical characterisation of their supercoiled DNA, their phage sensitivity and their inheritance of unselected markers, indicating that they conserve *Klebsiella* genetic material, including the *nif* operon, as CCC-DNA molecules.

METHODS

Bacteria. Characteristics and sources of bacterial strains are given in Table 1.

Bacteriophages. See Cannon *et al.* (1974).

Other procedures. Cannon *et al.* (1974) gave details of the procedures for culture, mating, acetylene tests for nitrogen fixation, isolation of clones resistant to phage EC1, assay and electrophoresis of gluconate-6-phosphate dehydrogenase, and labelling, extraction, centrifugation and counting of DNA.

Electron microscopy. DNA was prepared for electron microscopy essentially as described by Davis, Simon & Davidson (1971). A portion of 50 μ l of spreading solution, which contained 10 μ l of 0.1 % cytochrome *c*, 50 μ l of 1 M-ammonium acetate and 40 μ l of dialysed material from ethidium bromide-CsCl gradients, was spread on a hypophase of 0.25 M-NH₄ acetate. The film was picked up on grids coated with 3 % parlodion, stained with uranyl acetate and air dried. Grids were rotary shadowed with Pt-Pd (80:20) and viewed in an AEI EM 6G electron microscope. Micrographs were taken at magnifications from 6000 to 16 000, using a 50 μ m objective aperture and 60 kV accelerating voltage. Enlarged prints were traced on tracing paper and contour lengths of molecules measured with a map measurer. Molecular weights were estimated by measuring the contour length of colicin EI DNA prepared simultaneously with plasmids of hybrid C-L4, taking its molecular weight as 4.2×10^6 daltons and assuming proportionality to contour length.

RESULTS

Mobilization of Klebsiella markers in Escherichia coli hybrids

The presence of R144*drd*3 (the R factor used to promote transfer between *Klebsiella pneumoniae* and *E. coli*; Dixon & Postgate, 1972) in C-M9 and C-L4 was indicated by resistance to kanamycin, production of colicin I and sensitivity to phage If₂. The high transfer frequencies of kanamycin resistance to recipient strains (Table 2, Expts 1 and 4) showed that both hybrids possessed normal R144*drd*3 sex factor activity.

The determinants *his* and *met* G were not transferred readily by R144*drd*3 in hybrid C-M9 (Table 2, Expt 1). In contrast, these markers were mobilized by this R factor in hybrid C-L4 at high frequency (Table 2, Expt 4) compared with control matings (Table 2, Expt 6) with C603 (R144*drd*3) as the donor (C603 was the *Escherichia coli* recipient strain from which C-M9 and C-L4 were originally derived). In addition, *his* was transferred readily from C-L4 to SB1801 (a K12 strain with a complete deletion of the *his* operon).

The F-like R factor R1*drd*19 was introduced into both hybrids from *Escherichia coli* J53;

Table 1. *Bacterial strains and R factors*

Strain	Genotype	Bacteria	Other properties	Origin
<i>Klebsiella pneumoniae</i>				
HF3		Prototroph, Nif ⁺ ; derivative of M5a 1 (R144dvd3)		Dixon & Postgate (1972)
<i>Escherichia coli</i> K12				
J53	<i>pro met</i>	—	—	E. Meynell
W1166 (Col V)	—	Colicin I indicator	—	E. Meynell
SB1801	<i>his-750</i> (deletion of <i>his gnd</i> RHA-2A) <i>str A</i> λ^B <i>ara gal mal A xyl mil</i> (λ)— <i>thy thr leu thi</i>	Carries Col E1		P. Hartman
CR34 (Col E1)				D. Sheratt
<i>E. coli</i> C				
C603	<i>his arg str</i>	—	—	G. Bertani
CI463	<i>his-5 met G str-8 rha-2</i>	His ⁺ Nif ⁺ ; carries R144dvd3		G. Bertani
C-M9	<i>arg str</i>	His ⁺ Nif ⁺ ; carries R144dvd3		Dixon & Postgate (1972)
C-M91	<i>arg str</i>	His ⁺ Nif ⁺ ; carries R144dvd3		Segregant of C-M9
C-14	<i>arg str</i>	His ⁺ Nif ⁺ ; carries R144dvd3		Dixon & Postgate (1972)
R factors				
Designation	Determinants	Type		
R144dvd3	Km Col I	I		
R1dvd19	Km Am Cm Sm Su	F		
		Origin		
		E. Meynell		
		E. Meynell		

Genetic symbols for chromosomal markers are those of Taylor & Trotter (1972). 'Nif' denotes nitrogen fixation genes in accordance with the terminology of Streicher, Gurney & Valentine (1971). Symbols for drug resistance determinants are as follows: Km, kanamycin; Am, ampicillin; Cm, chloramphenicol; Sm, streptomycin; Su, sulphonamide.

Table 2. *Transfer characteristics of hybrids C-M9 and C-L4 carrying R144drd3 and R1drd19*

Frequencies expressed/donor organism. Km denotes resistance to kanomycin, Cm resistance to chloramphenicol. In certain experiments exconjugants were purified by streaking on the selective medium and then tested for inheritance of unselected markers. No. of colonies inheriting unselected marker/total exconjugants tested were: (a), Nif⁺ 28/30; (b), Nif⁺ 30/30; (c), Nif⁺ 30/30; (d), His⁺ 2/141; (e), Met⁻ 49/149.

Expt no.	Donor	Recipient						
		<i>E. coli</i> C1463				<i>E. coli</i> K12 SB1801		
		Km	Cm	<i>his</i>	<i>met</i> G	Km	Cm	<i>his</i>
1	C-M9(R144drd3)	1 × 10 ⁻¹	—	6 × 10 ⁻⁷	< 1 × 10 ⁻⁷	—	—	—
2	C-M9(R144drd3)(R1drd19)	—	> 1	7 × 10 ⁻⁴ (a)	3 × 10 ⁻⁴	—	1 × 10 ⁻¹	7 × 10 ⁻⁴ (b)
3	C-M91(R144drd3)(R1drd19)	—	1 × 10 ⁻¹	< 1 × 10 ⁻⁷	1 × 10 ⁻⁶	—	—	—
4	C-L4(R144drd3)	4 × 10 ⁻¹	—	5 × 10 ⁻⁴ (e)	7 × 10 ⁻³ (d)	2 × 10 ⁻³	—	2 × 10 ⁻³ (c)
5	C-L4(R144drd3)(R1drd19)	—	3 × 10 ⁻³	3 × 10 ⁻⁴	—	—	—	—
6	C603(R144drd3)	> 1	—	< 1 × 10 ⁻⁸	1 × 10 ⁻⁷	—	—	—
7	C603(R144drd3)(R1drd19)	—	1 × 10 ⁻¹	< 1 × 10 ⁻⁷	1 × 10 ⁻⁶	—	—	—

this sex factor behaved normally in both strains and transferred chloramphenicol resistance at the high frequencies expected for a derepressed R factor (Table 2, Expts 2, 3 and 5). R1drd19 mobilized *his* and *met* G in C-M9 at high frequency compared with control matings with C603 or C-M91, a His⁻ segregant of C-M9, as the donor (Table 2, Expts 2, 3 and 7). The presence of R1drd19 had little effect on the transfer frequency observed with C-L4 (Table 2, Expt 5). The above data indicate that the *his* and *met* G markers which presumably originated from Klebsiella are most probably plasmid borne in C-L4 and C-M9.

Linkage data

Exconjugants from various crosses were purified on selective media and screened for inheritance of unselected markers. With C-M9 as donor, 58 clones out of 60 His⁺ progeny were Nif⁺ [Table 2, crosses (a), (b)] whereas 30 His⁺ exconjugants from a mating with C-L4 were also Nif⁺ [Table 2, cross (c)]. This suggests that *his* and *nif* were linked in both hybrids. The *his* and *met* G determinants were evidently not linked in C-L4: when *met* G was the selected marker, only two out of 141 Met G⁺ exconjugants were also His⁺ and when *his* was selected only 49 out of 149 His⁺ exconjugants were Met G⁺ [Table 2, crosses (d), (e)].

Other non-selected markers inherited by hybrids

Phage resistance. Hybrids C-M9 and C-L4 were resistant to phages λvir, λh⁸⁰, P1vir and φX174. Resistance to the rough-specific phage, φX174, suggested that the *his*-linked *rfb* genes of Klebsiella were transferred to both hybrids (Cannon *et al.* 1974). Cannon *et al.* (1974) described the isolation and some characteristics of the hybrid-specific phage, EC1. Although hybrids C-M9 and C-L4 were sensitive to the phage, resistant variants arose at a high frequency. All of 163 EC1-resistant clones of C-M9 tested were His⁻ and Nif⁻.

Colony form. In contrast to a stable Nif⁺ hybrid, C-M7, both C-M9 and C-L4 produced mucoid colonies which suggests that they inherited genes affecting the production of extracellular polysaccharides. Since *his* was the selective marker for these hybrids, a locus in the Klebsiella donor equivalent to the *his*-linked *non* (non-mucoid) locus of *Escherichia coli* K12 (Radke & Siegel, 1971) could affect capsule synthesis in *E. coli* C.

Gluconate-6-phosphate dehydrogenase. Strain C-M7, with chromosomally integrated Klebsiella DNA, contained only one electrophoretically distinguishable form of this dimeric

Fig. 1

Fig. 2

Fig. 1. Fractionation of DNA in cleared lysates of *Escherichia coli* C603 (●) and hybrid C-M9 (○), grown in 20 ml cultures, after dye-buoyant density centrifugation at 126 000 g, 0 to 2 °C for 16 h. Clear lysates were prepared by a lysozyme-EDTA-Triton X-100 procedure (Cannon *et al.* 1974). After centrifugation, fractions (10 drops) were collected in styrene 'Microtiter' plates. A portion (0.01 ml) of each fraction was spotted on a filter disc and assayed for ^3H radioactivity.

Fig. 2. Profiles of DNA from hybrid C-M9, analysed by velocity centrifugation in neutral (a) and alkaline (b) 5 ml, 5 to 20% sucrose gradients. Fractions of the denser band from dye-buoyant density gradients were pooled and dialysed against TES-2 buffer. Portions (100 μl) of the pooled fractions, mixed with 100 μl Col E1 DNA solution were layered on gradients and centrifuged for 45 min (a) and 20 min (b) at 120 000 g in a 52 rotor at 20 °C using a Christ ωH ultracentrifuge. After centrifugation, seven-drop fractions were collected directly on to Whatman no. 3 filter discs and cold TCA precipitable label assayed for ^3H radioactivity.

enzyme, though the parent *Klebsiella* and *Escherichia* enzymes are distinguishable (Cannon *et al.* 1974). Polyacrylamide gel electrophoresis indicated three species of the enzyme in C-M9. The R_F values of two of these species corresponded to those of the enzyme from the parent *Klebsiella* (0.38) and *Escherichia* (0.47) strains. The third species had an intermediate R_F value (0.43) and was presumably a hybrid of one *Klebsiella* and one *Escherichia coli*

Table 3. *Molecular weights of circular DNA molecules from hybrid C-M9*

Molecular weight were calculated from $s_{20, w}$ values using the data of Bazaral & Helinski (1968).

$s_{20, w}$ of CCC molecules		Molecular weight (daltons)
Neutral sucrose	Alkaline sucrose	
77	208	69×10^6
97	254	118×10^6
33	104	9.5×10^6

monomeric subunit. This form was not observed when the parental enzymes were mixed *in vitro*. These results imply that C-M9 carried both parental *gnd* alleles.

Isolation and characterization of supercoiled DNA from hybrids

Cultures of *Eschichia coli* C603, hybrids C-M9 and C-L4, were labelled with tritiated thymidine and gently lysed by the lysozyme-Triton X-100 procedure to obtain cleared lysates (Cannon *et al.* 1974). These were then centrifuged in caesium chloride with ethidium bromide. DNA from both hybrids was resolved into two bands on the density gradients; the denser band was characteristic of CCC-DNA while the less dense band contained open circular (OC) and linear DNA (Fig. 1). CCC-DNA was not obtained from strain C603 under the experimental conditions used. Pooled fractions of the denser bands were dialysed against TES-2 buffer and analysed by velocity centrifugation as described by Cannon *et al.* (1974).

Hybrid C-M9. The combined results from neutral and alkaline sucrose gradients showed that C-M9 contained three species of CCC-DNA (Fig. 2*a, b*). One of the CCC-DNA species was R144*drd3*; it had S values of 77 for the CCC and 48 for the OC forms, the values established for this plasmid (Cannon *et al.* 1974). The remaining CCC-DNA species apparently arose from *Klebsiella* chromosomal DNA transferred to C-M9, since CCC-DNA was not observed in *Klebsiella pneumoniae* M5a1 and R144*drd3* was the only plasmid observed in its derivative donor strain, HF3 (unpublished). The S values of the plasmids formed in C-M9 were calculated from the S value of either Col E1 or R144*drd3* CCC-DNA; assuming a linear relationship between the S value and the distance sedimented, they were 97 S and 254 S for the CCC-form of the larger plasmid in neutral and alkaline gradients respectively, and 33 S and 104 S for that of the smaller plasmid. The molecular weights of these plasmids, calculated from the S values of their CCC-forms in neutral sucrose gradients using the data of Bazaral & Helinski (1968), were 118×10^6 and 9.5×10^6 daltons (Table 3).

The supercoiled DNA of an EC1-resistant segregant (C-M91) of C-M9, which was His⁻ Nif⁻, was examined in sucrose gradients (Fig. 3*a, b*). The CCC-DNA species from C-M9 of 33 S in neutral and 104 S in alkaline sucrose gradients was absent, though otherwise the profiles for C-M9 and C-M91 were identical. Hence C-M91 retained R144*drd3* and a larger plasmid which presumably originated from a segment of the *Klebsiella* chromosome. Therefore *his* and *nif* were probably located on a plasmid of molecular weight 9.5×10^6 daltons in C-M9.

Hybrid C-L4. Comparable combined results showed that C-L4 contained four species of CCC-DNA (Fig. 4*a, b*). The most rapidly sedimenting CCC-DNA species in neutral sucrose gradients had an S value of 92. In alkaline conditions this plasmid was centrifuged to the bottom of the gradient. A CCC species of 71 S in neutral sucrose and 208 S in alkaline sucrose was most probably R144*drd3*. Two additional rapidly sedimenting components characteristic of CCC-DNA were resolved in alkaline sucrose gradients. These plasmids (173 S and 112 S) partially overlapped with the 77 S and 48 S forms, respectively, of R144*drd3* in neutral sucrose gradients.

Fig. 3

Fig. 4

Fig. 3. Characterization of supercoiled DNA from an *Escherichia coli* Nif⁻ segregant, c-M91, by velocity centrifugation in 5 ml neutral (a) and alkaline (b) 5 to 20% sucrose gradients. Supercoiled DNA, purified by dye-buoyant density centrifugation, was dialysed against TES-2 buffer and portions (100 μ l of c-M91 DNA and 100 μ l of Col E1 DNA) layered on sucrose gradients. Centrifugation was carried out for 45 min (a) and 20 min (b) at 120 000 g in a 52 rotor at 20 °C using a Christ ω II ultracentrifuge. Gradients were fractionated by drop collection (7 drops/fraction) directly on to Whatman no. 3 filter discs and cold TCA precipitable label assayed for ³H radioactivity.

Fig. 4. Profiles of DNA from hybrid c-L4, analysed by velocity centrifugation in neutral (a) and alkaline (b) 5 ml, 5 to 20% sucrose gradients. Supercoiled DNA, obtained from dye-buoyant density gradients, was dialysed against TES-2 buffer and portions (100 μ l of c-L4 DNA and 100 μ l of Col E1 DNA) layered on sucrose gradients. Centrifugation was carried out for 45 min (a) and 30 min (b) at 120 000 g in a 52 rotor at 20 °C using a Christ ω II ultracentrifuge. Gradients were fractionated by drop collection (7 drops/fraction) directly on to filter discs and TCA precipitable label assayed for ³H radioactivity.

(a)

(b)

(c)

(d)

Fig. 5. Electron micrographs of circular DNA from hybrid C-L4, purified by dye-buoyant density centrifugation, showing members of four classes with mean Col E1 DNA equivalents of (a) 24.8, (b) 14.5, (c) 11 and (d) 2.8. Bars represent 1 μ m.

Table 4. *Molecular weights of circular DNA molecules from hybrid C-L4*Molecular weights were calculated from $s_{20,w}$ values and counter-length measurements.

$s_{20,w}$ of CCC molecules		Molecular weight* (daltons)	No. of molecules measured	No. of E1 DNA equivalents	Molecular weight† (daltons)
Neutral sucrose	Alkaline sucrose				
—	208	69×10^6	20	14.5 ± 0.25	61×10^6
92	—	106×10^6	6	24.8 ± 0.3	104×10^6
—	173	—	8	11 ± 0.21	46×10^6
—	112	—	28	2.8 ± 0.1	12×10^6

* Calculated from $s_{20,w}$ values using the data of Bazaral & Helinski (1968).† Calculated from contour lengths assuming a molecular weight of 4.2×10^6 daltons for Col E1 DNA.*Electron microscopy*

Electron micrographs of plasmids from hybrid C-L4 are shown in Fig. 5 and measurements of contour lengths are presented in Table 4. Four distinct sizes were observed among the molecules examined, corresponding to the four sedimentation types. The molecular weights calculated from the contour lengths (see Methods) agreed well with those derived from sedimentation coefficients.

DISCUSSION

In contrast to hybrid C-M7, where a fragment of *Klebsiella* DNA is probably integrated into the *Escherichia coli* chromosome (Cannon *et al.* 1974), the two hybrids studied here harboured multiple plasmids which were apparently derived from the *Klebsiella* chromosome (see below). The presence of three plasmids in hybrid C-M9 and four in C-L4 made their assignment difficult. Unfortunately, we were unable to purify sufficient DNA of each plasmid for use in transformation experiments, and since the hybrids were resistant to transducing phage we were also unable to study the plasmids by transduction. Therefore, although it was possible to characterize these plasmids physically using sucrose density gradients and electron microscopy, it was not feasible to assign a function to each CCC molecule unequivocally.

Both hybrids carried a plasmid identifiable as R144drd3, the R factor originally introduced into the donor strain of *Klebsiella pneumoniae* (Dixon & Postgate, 1971). Strain C-M9 contained a plasmid of molecular weight 9.5×10^6 daltons whose loss in C-M91 was accompanied by loss of *nif* and other *his*-linked characters; this plasmid presumably carried *nif* and associated determinants. The presence of three classes of gluconate-6-phosphate dehydrogenase in hybrid C-M9 is unequivocal evidence for duplication of the *gnd* locus in this hybrid; presumably the *Klebsiella gnd* determinant is also carried on this plasmid because *gnd* maps close to *nif* (Cannon *et al.* 1974).

The corresponding plasmid in C-L4 was not identified in this way; it may be the one of molecular weight 12×10^6 daltons. The ready mobilization of *his* and *nif* by R144drd3 in this strain provides evidence that these determinants are plasmid-borne and contrasts with the situation in C-M9 in which R144drd3 did not mobilize efficiently. The *met* G determinant in hybrid C-L4, which presumably originated from *Klebsiella*, was transferred at high frequency by R144drd3 and was not linked to *his*. The *Klebsiella met* G determinant may therefore be carried on another of the three plasmids identified in this hybrid.

A consequence of the formation of *nif*-bearing plasmids in these hybrids is that gene transfer is no longer hindered by problems arising from differences in nucleotide sequence

homology and host-specific restriction. Hence numerous Nif⁺ strains of *Escherichia coli* K12 were obtained (Table 2), which could not reasonably have been expected from a direct mating with *Klebsiella pneumoniae* unless a mutant deficient in host-specific restriction were chosen. The primary genetic barrier to more widespread transfer of the *nif* operon is now the host range of existing sex factors and their ability to mobilize the *nif*-bearing plasmid. In many organisms, however, the reduced conditions required for expression of *nif* (see Postgate, 1971) may present a phenotypic obstacle to obtaining nitrogen-fixing strains in practice.

The presence of multiple plasmids derived from *Klebsiella* chromosomal DNA in the two hybrids contrasts with the situation in the *Escherichia*-*Salmonella* hybrids studied by Leavitt *et al.* (1971). Each *Salmonella* hybrid contained only one plasmid, though among the hybrids a wider range of plasmid molecular sizes occurred; the upper range of size of the *Salmonella* plasmids was considerably larger than that in our *Escherichia coli* hybrids. The origin of the plasmids in *E. coli* is a complex question. They did not exist as free plasmids in the original donor strain of *Klebsiella* (*Klebsiella pneumoniae* strain HF3; Dixon & Postgate, 1972) because no plasmid other than R144drd3 could be detected in this strain; nor could a plasmid be detected in the recipient strain *E. coli* C603. Presumably large segments of *Klebsiella* DNA were transferred, but whether these fragmented before forming several CC circles or whether a large circle was formed which looped off smaller circles is not known. Since each plasmid presumably originated from a segment of the *Klebsiella* chromosome it is difficult to visualize how each DNA molecule is able to replicate autonomously, and the establishment of new replicons in each hybrid is an extremely vexing problem. The DNA segments may have gained the ability to replicate by recombination with cryptic replicons which cannot be detected by our physico-chemical procedures. Alternatively, the *Klebsiella* chromosome itself may contain redundant replicons which became expressed after transfer to *E. coli*.

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