Acta Biochimica Polonica

Vol. 46 No. 4/1999

853-861

QUARTERLY

Plasmid-mediated suppression of the mutational activation of the *bgl* operon in *Shigella sonnei*[°]

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Received: 29 October, 1999

Key words: β-glucosides, *Shigella sonnei*, extrachromosomal regulation, *ColE1* like plasmids, mutational activation

SSOR, a clinical isolate of *Shigella sonnei* which exhibits a Salicin-negative phenotype, is unable to mutate to give rise to Sal$^+$ derivatives although a homolog of the *Escherichia coli bgl* operon is retained by the strain. This was correlated to the presence of an endogenous plasmid in the strain. A plasmid-cured derivative, AK711, could give rise to Sal$^+$ mutants in two steps. Introduction of the plasmid DNA, extracted from SSOR, into various strains of *E. coli* and *S. sonnei*, resulted in ampicillin resistant transformants. Interestingly, the presence of the plasmid suppressed the mutational activation of the *bgl* operon in the transformants. This was further substantiated by the observation that, transformants that have lost the plasmid regained the ability for mutational activation of the *bgl* operon. Preliminary characterisation of the plasmid indicated a size of 3.8 kb with an origin of replication resembling that of *ColE1* replicons and the *bla* gene homolog of Tn*3*. Observations of the mutation frequency at the *srl* and *lac* loci in the presence of the plasmid indicate that there is a reduction in the mutation frequency, suggesting an antimutator activity associated with the plasmid.

The ability to utilize aryl β-glucosides such as salicin, arbutin, and cellobiose, varies markedly among members of the family *Enterobacteriaceae* (Schaefler & Malamy, 1969). One of the interesting aspects of β-glucoside utilization in *Escherichia coli* is that all the four different systems involved are cryptic and require mutational activation (Hall & Betts, 1987; Hall & Xu, 1992; Kricker & Hall, 1987; Mukerji & Mahadevan, 1997 – for a review; Parker & Hall, 1988). Of these four systems, mutational activation of the *bgl* operon (conferring the ability to transport and metabolize arbutin and salicin) occurs most frequently.

[°]This work was supported by a grant to S.M. from the Council for Scientific and Industrial Research, Government of India.

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The *bgl* operon of *E. coli* is a paradigm for cryptic genetic systems. Though wild type cells are phenotypically Bgl$^-$, a single mutational event can lead to the simultaneous acquisition of a Salicin$^+$ Arbutin$^+$ phenotype (Prasad & Schaefler, 1974; Reynolds *et al.*, 1981). Mutations that activate the operon map predominantly within the regulatory region *bglR* and in most cases are caused by insertion of IS elements upstream of the promoter leading to enhancement of transcription from the *bgl* promoter (Reynolds *et al.*, 1981; 1986). Transcriptional activation is mediated by the disruption of negative elements located near the promoter (Lopilato & Wright, 1990; Mukerji & Mahadevan, 1997; Schnetz, 1995; Singh *et al.*, 1995). Activation results in the expression of a phospho-*enol*-pyruvate (PEP)-dependent phosphotransferase (encoded by the *bglF* gene) and a phospho-β-glucosidase B (encoded by *bglB)* which can cleave salicin and arbutin (Prasad & Schaefler, 1974). In addition *E. coli* constitutively expresses the enzyme phospho-β-glucosidase A, specific for arbutin, encoded by the unlinked *bglA* locus (Prasad *et al.*, 1973).

In addition to the two structural genes *bglB* and *bglF*, the operon also encodes a positive regulator *bglG*. BglG mediates transcriptional regulation *via* antitermination of transcription at two rho-independent terminators flanking *bglG* (Mahadevan, 1997; Rutberg, 1997 for a review).

Earlier biochemical studies on five different isolates of *Shigella sonnei* have shown that they fall into two classes, one capable of mutating to a Sal$^+$ phenotype in two steps (Class I) and another that is unable to mutate to Sal$^+$ even after prolonged incubation (Class II) (Schaefler & Malamy, 1969). To date, no genetic or molecular information is available that can provide a satisfactory explanation for these differences. Functional and molecular studies have shown that both Class I and Class II strains of *S. sonnei* carry a homolog of the *E. coli bgl* operon (Kharat & Mahadevan, submitted for publication). In this report, we present evidence that the presence of an endogenous plasmid in the Class II strains suppresses the mutational activation of the *bgl* genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Plasmid

Table 1. Bacterial strains

Strain	Genotype or Description	Source or Reference
E. coli K-12		
RV	F$^-$ Δ*lac*X74 *thi* *bglR*^{0} *bglG*$^+$ *bglF*$^+$ *bglB*$^+$ (Arb$^-$ Sal$^-$)	A. Wright
JF201	F$^-$ Δ*lac*X74 Δ(*bgl-pho*)201 *ara thi gyrA*	Reynolds *et al.*, 1986
DH5α	F′/*endA1 hsdR17* (rk$^-$ mk$^-$)*SupE44 thi-1recA1 gyrA*(Nal^R) *relA1* Δ(*lacZYA-argF*) *U169deo*R(ϕ80d*lac*Δ(*lacZ*)M15)	Woodcock *et al.*, 1989
S. sonnei		
AK1	*bglR*^{0} *bglG*$^+$ *bglF*$^+$ *bglB*$^-$ (Arb$^-$ Sal$^-$)	this work
SSOR	*bglR*^{0} *bglG*$^+$ *bglF*$^+$ *bglB*$^-$ (Arb$^-$ Sal$^-$)Amp^R Tet^R Kan^R	R. Roy
AK711	Plasmid-cured derivative of SSOR Amp^S Tet^R Kan^R	this work
AK811	Arb$^+$ derivative of AK711 (Arb$^+$ Sal$^-$) carrying pMN5(*bglF*$^+$)	this work
AK811-1	Sal$^+$ derivative of AK811 (Arb$^+$ Sal$^+$)	this work
AK916	Arb$^+$ derivative of AK711 (Arb$^+$ Sal$^-$)	this work
AK916-6	Sal$^+$ derivative of AK916 (Arb$^+$ Sal$^+$)	this work

curing and isolation of mutants is detailed below.

Plasmid curing. Strain SSOR was cured of the endogenous plasmid using acridine orange by two methods. In one experiment, overnight cultures were grown in LB medium supplemented with 25 μg/ml acridine orange. Cultures from stationary phase were diluted in physiological saline. Appropriate dilutions were plated on LB agar and single colonies obtained were patched on plates containing 100 μg/ml of ampicillin to screen for loss of ampicillin resistance. In another approach, an overnight culture was diluted in physiological saline. Appropriate dilutions were plated on LB agar supplemented with 25 μg/ml acridine orange. Single colonies obtained were checked for loss of ampicillin resistance as before. The strain AK711, described in this study, was obtained by the former method.

Table 2. Plasmids

Plasmid	Vector	Description	Source or Reference
pMN5	pBR322	*Bgl'G bglF*$^+$*bglB'*	Mahadevan & Wright, 1987
pBR322	-	Amp^{R}Tet^R *colE1*	Bolivar *et al.*, 1977
pLG339	-	Kan^{R}Tet^R *psc105*	Stoker *et al.*, 1982
pANK3	-	Amp^R *colE1*	this work

Plasmid-cured derivatives of SSOR were also obtained after 12 rounds of successive passages on LB agar. Clones showing ampicillin-sensitive phenotype were reconfirmed as before and used for papillation studies.

Isolation of* Bgl$^+$ *mutants of SSOR. The strain AK711, a plasmid cured derivative of SSOR, (Arb$^-$ Sal$^-$) was streaked on MacConkey Arbutin plates and incubated at 37°C for 72 h to allow papillation. Arb$^+$ strains purified from the papillae remained Sal$^-$ on MacConkey Salicin plates. The Arb$^+$ Sal$^-$ strains were streaked on MacConkey Salicin plates and were incubated at 37°C for 120 h to allow papillation. Arb$^+$Sal$^+$ derivatives were purified from the papillae. Approximate mutation frequencies were calculated from the papillation frequencies. Sorbitol$^+$ and Lactose$^+$ were scored similarly after incubation on MacConkey Sorbitol and Lactose plates, respectively.

DNA manipulations. Plasmid isolation, DNA manipulations, and Southern analysis were carried out as described previously (Sambrook *et al.*, 1989). Isolation of genomic DNA was carried out, with minor modifications according to published protocol (Owen & Borman, 1987). Plasmid DNA was introduced into cells by transformation.

RESULTS

Genetic analysis of SSOR

Among four different strains of *S. sonnei* tested for β-glucoside utilisation, three could papillate on MacConkey's Arbutin plate with a frequency of 10^{-8} within 72 h of incubation at 37°C. Arb$^+$ mutants, purified from the papillae, could utilize arbutin but remained Sal$^-$. The Arb$^+$ phenotype is the result of transcriptional activation of the *bgl* operon (Kharat & Mahadevan, submitted for publication). However, the strain SSOR, one of the clinical isolates, could not papillate on MacConkey's Arbutin or MacConkey's Salicin plate even after prolonged incubation of 10–15 days at 37°C. One possibility is that the class II strains carry a mutation in the *bglF* gene encoding the permease. To rule out this possibility, the strain SSOR was cured of the plasmid encoding resistance to ampicillin as described in Materials and Methods. One such ampicillin-sensitive strain, AK711, was transformed with the plasmid pMN5 carrying the *E. coli bglF* gene. The transformant could papillate on MacConkey's Arbutin plates. Interestingly, the strain AK711 itself could papillate with a frequency of 10^{-8} on Mac-

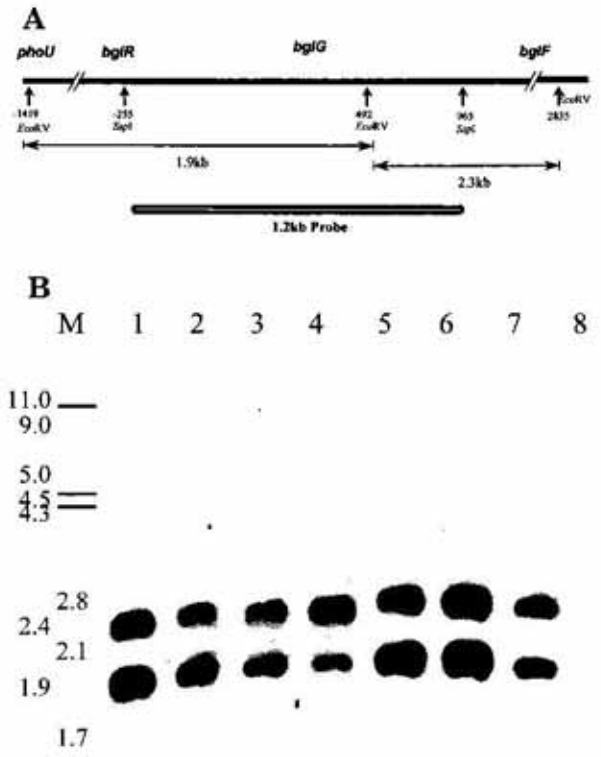

Figure 1. Comparison of the organisation of the *bgl* genes in Class I and Class II strains of *S. sonnei*.

A. Organisation of the *bglR*, *bglG* and *bglF* loci in *S. sonnei* Class I strains. Restriction enzyme sites are indicated by vertical arrows. The probe used in the characterisation of the *bglR* region is shown as an open box.

B. Southern analysis of *S. sonnei* genomic DNA digested with *Eco*RV, using the 1.2 kb *Ssp*I probe spanning the *bglR–bglG* region.

Lane 1, SSOR – WT *S. sonnei* strain-Class II (Arb^-Sal^-); Lane 2, AK711 – plasmid cured derivative of SSOR (Arb^-Sal^-); Lane 3, AK811-Arb^+ derivative of AK711 carrying pMN5-*bglF*$^+$ (Arb^+Sal^-); Lane 4, AK811-1 – Sal^+ derivative of AK811 (Arb^+ Sal^+); Lane 5, AK916 – Arb^+ derivative of AK711 (Arb^+ Sal^-); Lane 6, AK916-6 – Sal^+ derivative of AK916 (Arb^+ Sal^+); Lane 7, AK1 – WT *S. sonnei* strain-Class I (Arb^-Sal^-); Lane 8, JF201 (Δ*bgl* strain of *E. coli*).

Conkey's Arbutin plates and Arb^+ derivatives purified from papillae could mutate to Sal^+. The mutation frequencies were similar to class I strains of *Shigella sonnei*. This result suggests that the lack of papillation in the strain SSOR can be correlated to the presence of the endogenous plasmid. Plasmid curing efficiency was 88% with respect to the loss of the ampicillin marker, suggesting curing of small molecular mass plasmid.

To rule out the possibility that the papillation seen in the strain AK711 is the result of an alteration produced by acridine orange treatment, a plasmid-cured derivative of SSOR, obtained after serial passage in the absence of selection, was tested for the ability to papillate on MacConkey's Arbutin plates. The results obtained were similar to that seen in the earlier experiment. The only difference was a 10-fold reduction in the papillation frequency in this case. These results confirm that the papillation seen in the plasmid-cured derivative is not an artifact of acridine orange treatment of the cells. The difference in the papillation frequency in the two cases may be related to the loss of additional extra-chromosomal elements during acridine orange treatment, which may contribute to the reduction in the mutational activation.

Identification of the *bgl* homolog in SSOR

Since the strain SSOR could not be activated to give rise to Bgl^+ derivative even after prolonged incubation, one possibility is that there are structural differences in the *bgl* genes carried by Class I strains of *S. sonnei*. To test this possibility, the *bgl* homologs of Class I and Class II strains were examined by Southern analysis using the *E. coli bgl* genes as a probes. These studies, shown in Figs. 1 and 2, indicated that the organization of the *bgl* operon is similar in both classes of strains. The size of the restriction fragment corresponding to the *bglR* region in the Arb^+ and Sal^+ strains is similar to that of SSOR, indicating that the activation of the operon in the Arb^+ and Sal^+ strains is not because of transposition of IS element within the *bglR* region. Activation in these strains is the result of mutations at unlinked loci (Kharat & Mahadevan, unpublished).

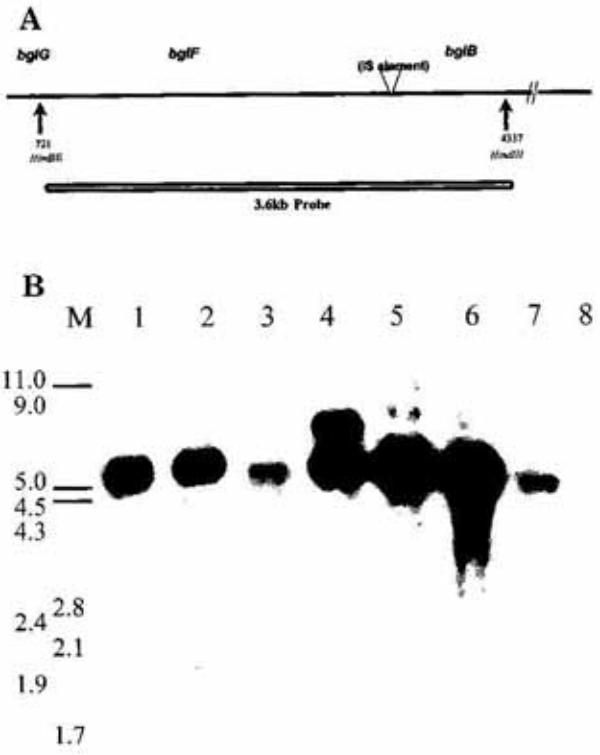

Figure 2A. Restriction map of the *bglF* and *bglB* region present in *S. sonnei* Class I strains.

The *Hin*dIII restriction enzyme sites are indicated by vertical arrows. The 3.6 kb *Hin*dIII probe made from *E. coli* plasmid used in the Southern analysis of the *bglF–bglB* region is shown as an open box.

Figure 2B. Southern analysis of the *bglF* and *bglB* region of *S. sonnei*.

Lane 1, SSOR– WT *S. sonnei* strain-Class II (Arb^-Sal^-); Lane 2, AK711 – plasmid cured derivative of SSOR (Arb^-Sal^-); Lane 3, AK811-Arb^+derivative of AK711 carrying pMN5-*bglF*$^+$(Arb^+Sal^-); Lane 4, AK811-1 – Sal^+ derivative of AK811 (Arb^+Sal^+); Lane 5, AK916 – Arb^+ derivative of AK711 (Arb^+Sal^-); Lane 6, AK916-6 – Sal^+ derivative of AK916 (Arb^+Sal^+); Lane 7, AK1 – WT *S. sonnei* strain-Class I (Arb^-Sal^-); Lane 8, JF201 (Δ*bgl* strain of *E. coli*).

Characterisation of the endogenous plasmid from SSOR

Since plasmid-cured strains of SSOR could mutate to Sal^+ in two steps, attempts were made to characterize the plasmid present in SSOR. Plasmid DNA isolated from SSOR was used to transform *E. coli* K-12 strains. Transformants were selected on ampicillin plates. DNA extracted from Amp^R transformants was used for restriction analysis. The pattern shown by plasmid isolates from 50 different transformants was similar. The plasmid pANK3, isolated from one of the transformants, was used for further characterization. The results of these studies indicated a plasmid size of 3.8 kb. Dot blot using pANK3 DNA was used to identify the gene encoding ampicillin resistance and the origin of replication of the plasmid (Bolivar *et al.*, 1977; Goransson *et al.*, 1990; Stoker *et al.*, 1982; Stucliffe, 1987). The results of this analysis, presented in Fig. 3A and 3B, showed that the *bla* and *ori* probes derived from pBR322 hybridized to pANK3 DNA, suggesting that pANK3 is a *colE1* replicon carrying the Tn*3* derivative of the β-lactamase gene (Bolivar *et al.*, 1977; Stucliffe, 1987). Quantitative hybridisation analysis (Olsen, 1999) showed that pANK3 is a multicopy plasmid (not shown).

Effect of pANK3 on the papillation frequency

To investigate whether pANK3 has any effect on papillation frequency in different wildtype strains of *S. sonnei* and *E. coli*, the plasmid was introduced in the *S. sonnei* strains AK1 (sewage isolate) and AK711 and the *E. coli* strain RV with pBR322 as a control. Ampicillin-resistant transformants were patched on MacConkey's Arbutin plate and papillation was monitored. The results, presented in Table 3, showed that in the presence of pANK3, papillation frequency is reduced by about 20-fold in the case of *E. coli* and 15-fold in the case of *S. sonnei*. These observations indicate that the presence of pANK3 causes a drastic reduction in papillation frequency, but it is not reduced completely as in the original strain SSOR. This suggests that there may be additional plasmids present in SSOR that may contribute to the reduction in the mutational activation.

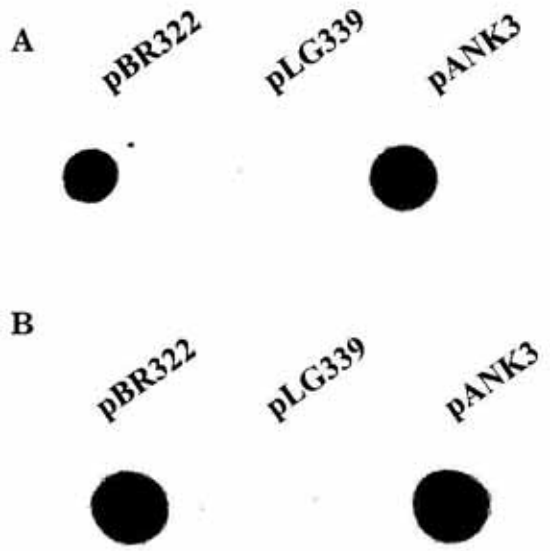

Figure 3A. Analysis of the locus conferring ampicillin resistance in pANK3.

Dot blot was prepared using DNA from pBR322 (positive control), pLG339 (negative control) and pANK3 (test DNA). The blot was hybridised to a α-^{32}P-labelled 700 bp *Pst*I-*Eco*RI fragment from the *bla* gene of pBR322.

Figure 3B. Analysis of the origin of replication in pANK3 by DNA dot blot.

DNA dot blot was made from the same set of plasmids as described above. The blot was hybridised to a α-^{32}P-labelled 600 bp *Alw*N1-*Bst*Z17 fragment from the origin region of pBR322.

To determine the effect of the plasmid pANK3 on strains that have been activated, the plasmid was introduced into Bgl$^+$ (instead of Sal$^+$) strains of *E. coli* and *S. sonnei*. Presence of the plasmid in these strains did not have any effect on the *bgl* phenotype of these strains, indicating that the plasmid did not affect the expression of the operon *per se*, but influenced only the mutational activation of the silent operon.

Table 3. Effect of pANK3 on the emergence of Arb$^+$ papilla on MacConkey's Arbutin plate

Strain	Plasmid	Papillation frequency $\times 10^8$
S. sonnei		
AK1	pANK3	9
AK1	pBR322	142
AK711	pANK3	10
AK711	pBR322	147
E. coli K-12		
RV	pANK3	10
RV	pBR322	200

Table 4. Effect of pANK3 on the emergence of Srl$^+$ and Lac$^+$ papilla on MacConkey's Sorbitol and MacConkey's Lactose plate, respectively

Strain	Plasmid	Papillation frequency $\times 10^8$	
		Srl$^+$	Lac$^+$
AK1	pANK3	7	6
AK1	pBR322	134	146
AK711	pANK3	6	7
AK711	pBR322	152	172
SSOR	-	ND*	ND*

*ND, Not detectable

In an attempt to determine whether the effect of the plasmid is specific to the *bgl* operon or is a general effect on mutations in the strain, the frequency of mutations occurring at other loci was tested in the presence and absence of the plasmid. Strains of *Shigella sonnei* show a Sorbitol$^-$, Lactose$^-$ phenotype, but can mutate to Srl$^+$ and Lac$^+$ independently, though the nature of these reversions have not been characterized. As in the case of *bgl* activation, reversion to Srl$^+$ and Lac$^+$ was measured in the strains AK1 and AK711 in the presence of pANK3 and pBR322 as control. The results, presented in Table 4, show that the mutation frequency is reduced in both cases in the presence of pANK3. Therefore, the plasmid has a general effect on lowering mutation frequency of the host.

DISCUSSION

The experiments described above were undertaken to understand the marked difference in Class I and Class II strains of *S. sonnei* with respect to the mutational activation of the *bgl* genes. Although the organization of

the *bgl* genes in both classes of *S. sonnei* is identical, the absence of papillation in Class II strains is an enigma. The one major discernable difference between the two classes is the presence of a small molecular mass plasmid conferring resistance to ampicillin in the Class II strains. Interestingly, the plasmid-cured derivative of the Class II strain SSOR, is capable of mutational activation of the *bgl* genes. In addition, our results also indicate a 15–20-fold decrease in papillation frequency when the plasmid is introduced in wild type strains. These results suggest a strong correlation between the presence of the plasmid and the suppression of papillation.

How does the endogenous plasmid influence the mutational activation of the *bgl* operon in Class II strains? One possibility is that presence of the active copy of the *bgl* operon leads to the expression of gene(s) present on the plasmid that is detrimental to the cell. As a result, any mutation that arises in the presence of the plasmid will be selected against. Alternatively, products of genes present on the plasmid may interfere with the expression of the *bgl* operon, either at the transcriptional or post-transcriptional level, resulting in a Bgl^- phenotype even when the operon has been mutationally activated. Because of this, Bgl^+ papillae cannot be detected. A third possibility is that in the presence of the plasmid, mutation frequency is lowered so that activation of the *bgl* operon occurs at a reduced frequency. Interestingly, when the plasmid pANK3 was introduced in a Bgl^+ strain of *S. sonnei*, there is no apparent reduction in the transformation frequency compared to control. In other words, the plasmid is tolerated in a strain that is already activated for *bgl* expression. In addition, the strain continues to exhibit a Bgl^+ phenotype. These results indicate that the possibility of the activated *bgl* operon stimulating the expression of detrimental genes carried on the plasmid is unlikely. As the transformants continue to exhibit a Bgl^+ phenotype, it is also unlikely that the plasmid-encoded genes suppress expression of the operon at the transcriptional or post-transcriptional level. This leaves us with the possibility that mutation frequency may be reduced in the presence of the plasmid. Measurements of reversion frequency at the *srl* and *lac* loci indicate that the effect of the plasmid is a general one. However, the mechanism of suppression of mutation is not clear at this stage. It is also necessary to identify the specific regions of the plasmid that are involved in the suppression. Molecular characterization of the locus/loci involved, including complete nucleotide sequence determination, can provide better insights on the mechanism of suppression.

There are several reports in the literature about genes encoded by megaplasmids such as pINV/or pINC, their positive and negative role in pathogenesis in *Shigella* (Sakai *et al.*, 1988; Sansonetti *et al.*, 1982; 1983; Winans & Walker, 1985). Loss of plasmid or deletions within the plasmid lead to the production of avirulent strains that are unable to penetrate and multiply within epithelial cells. Many times, under nonselective conditions, genes from the megaplasmid are known to integrate with the chromosome, resulting in negative regulation of the pathogenesis cascade (Zangaglia *et al.*, 1991). A majority of the pathogenic genes are located on the plasmid amongst which some interact with the host chromosome to facilitate infection. Hence for the establishment of pathogenesis, the co-ordinate expression of both plasmid and chromosomal genes is essential (Zangaglia *et al.*, 1991).

Suppression of crown gall formation in plants was shown to be mediated by a plasmid-coded protein. The IncW plasmid pSa, originally derived from *Shigella flexneri*, completely inhibited the tumor-inducing ability of *Agrobacterium tumefaciens* when it is resident in this organism (Chen *et al.*, 1994; Chernin *et al.*, 1984). Oncogenic inhibition was mediated through the expression of the *osa* gene on pSa. This gene is part of 3.1 kb

DNA segment of pSa that contains four open reading frames. Further characterization showed that the inhibition of oncogenicity of *A. tumefaciens* by the *osa* gene product is similar to the inhibition of conjugal transfer by a cohabiting plasmid (Chen *et al.*, 1994; Chernin *et al.*, 1984).

Interestingly, there are no reports about genes located on small plasmids influencing the regulation of catabolic operons. In this respect, pANK3 differs from the plasmids mentioned above both in its size as well as its possible mode of action. The physiological significance of this suppression is also not known at this point of time. The ability to conditionally alter mutation rates can have a major impact on the evolution of microorganisms in response to various selective pressures. This may also have an impact on adaptive mutagenesis reported in the case of several microorganisms.

We thank R. Roy and V. Ramchandran (ASTRA-Zeneca) for the gift of the SSOR strain and A. Wright for fruitful discussions.

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