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Identification of Two New Genetically Active Regions Associated with the *osmZ* Locus of *Escherichia coli*: Role in Regulation of *proU* Expression and Mutagenic Effect at *cya*, the Structural Gene for Adenylate Cyclase

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The *Escherichia coli* K-12 gene coding for the nucleoid-associated protein HNS was cloned together with 5.6 kb of downstream DNA in the vector pACYC184. The cloned DNA complemented a mutation in the *osmZ* locus of *E. coli*, which codes for HNS. However, the multicopy plasmid harboring the cloned sequence was found to be mutagenic and to produce at high frequency mutations that mapped to the *E. coli cya* gene, which codes for adenylate cyclase. Acquisition of the *cya* mutations was independent of RecA. These mutations were phenotypically suppressed by providing the cells with exogenous cyclic AMP and were complemented in *trans* by a plasmid carrying an active copy of the *cya* gene. A deletion analysis of the cloned sequences showed that DNA downstream of the gene coding for HNS was also required for the mutagenic effect at *cya* and had a role in regulating the expression of the *osmZ*-dependent *proU* locus. These sequences appear to contain at least two genetically active regions.

In enteric bacteria, the abundant, nucleoid-associated 15.4-kDa basic protein HNS (also known as H1 or B1) binds to and strongly compacts DNA (25, 48, 50). Data from isoelectric focusing experiments have shown it to exist in *Escherichia coli* K-12 in three forms, a, b, and c, with the DNA-compacting "a" form predominating in the stationary phase (48). The gene coding for HNS was originally designated *hns* and mapped to 6.1 min on the *E. coli* genetic map (38), although this map location was found subsequently to be incorrect (26, 34). The correct location of the gene coding for HNS is at the *osmZ* locus at 27 min on the *E. coli* genetic map and at 34 min on that of *Salmonella typhimurium* (2, 24–26). Homologs of *osmZ* have been cloned from several enteric bacteria, and the primary sequences of both the gene and gene product are highly conserved within this group (26, 30, 38).

The *osmZ* locus derives its designation from its genetically determined role in modulating the osmotic induction of the *proU* operon in *E. coli* and *S. typhimurium*. This operon encodes an uptake system for the osmoprotectant glycine-betaine, and transcription of *proU* is induced when cells are grown in high-osmolarity media (12, 24). Transcription of *proU* is under complex control and is partly regulated by negative supercoiling of DNA, at least when the *proU* promoter is in its native chromosomal context (24). Significantly, osmotic stress alters the level of negative supercoiling in bacterial DNA and is one of a number of environmental signals which have this effect. Others include anaerobic growth and changes in temperature, growth phase, or carbon source (4, 15, 16, 19, 51). There is accumulating evidence that bacteria exploit these changes in DNA topology to alter coordinately the expression of genes required to adapt to environmental change (14, 23, 37). Significantly, point mutations and transposon insertion mutations in *osmZ* cause changes in DNA supercoiling of a similar magnitude to those produced by environmental changes which alter the expression of genes such as *proU* (24), and it is believed that *osmZ* contributes to *proU* regulation by modulating the topology of the DNA in the environs of the *proU* promoter (24, 25).

Mutations in *osmZ* are highly pleiotropic, affecting the expression of several genes in addition to *proU* (21, 25). Indeed, the *osmZ* locus has been detected and named independently by several groups working on diverse genetic systems, and this has resulted in a complex nomenclature. Although the current edition of the *E. coli* genetic map gives priority to *osmZ* (2), the locus is also known as *bglY* (a negative regulator of the DNA supercoiling-sensitive *bgl* operon), *drdX* (contributing to the thermoregulation of Pap fimbriae in *E. coli*), *pilG* (a negative regulator of type 1 fimbriae in *E. coli*), and *virR* (contributing to the thermoregulation of plasmid-encoded invasion genes in *Shigella flexneri*) (13, 16, 20, 24, 27, 33, 49).

Several investigators have reported that multiple copies of *osmZ* are not well tolerated by *E. coli* and that when expressed from plasmids, the locus can cause alterations in cell morphology (26, 34). This is consistent with the notion that HNS is a major component of the nucleoid and perhaps plays a role in the regulation of the bacterial cell cycle. Furthermore, deletion mutations in *osmZ* are themselves mutagenic, promoting deletion mutations at other sites on the chromosome, possibly as a result of changes in chromatin structure (31).

In this study the effects of multiple copies of the *E. coli osmZ* locus on expression of the DNA supercoiling-sensitive *ara* (45, 46), *lac* (5, 6, 29, 44), and *proU* (24) operons were investigated. Because the complex nomenclature in the *osmZ* literature outlined above has contributed to confusion about the precise nature of this genetic locus, in this paper the DNA sequence coding for HNS is referred to as *hns*, as originally used by Pon et al. (38). The designation *osmZ* is reserved for the locus which includes *hns* and which affects expression of the osmotically regulated *proU* operon. We

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TABLE 1. Bacterial strains, bacteriophage, and plasmids

Strain, phage or plasmid	Relevant genotype/phenotype	Source or reference
E. coli K-12		
294	Wild type	3
294*recA*	*recA1*	This laboratory
BRE2076	MC4100 Φ(*proU-lacZ*)hyb2(λ*plac* Mu15) *osmZ203*	24
BW6159	*ilv-691*::Tn*10 relA1 spoT1 thi-1* Hfr PO68	32
CJD512	DH1(pGB619)/Ara$^-$ Lac$^-$	This study
CJD542	GM37(pGB619)	This study
CJD543	DH1(pACYC184)	This study
CJD546	GM37(pACYC184)	This study
CJD547	BRE2076(pGB619)	This study
CJD548	BRE2076(pACYC184)	This study
CJD552	CJD512(pGB619 pDIA100)	This study
CJD553	GM37(pGB634)/Lac$^+$	This study
CJD554	GM37(pGB634)/Lac$^-$	This study
CJD555	BRE2076(pGB634) Lac$^+$	This study
CJD559	GM37(pGB636)/Lac$^+$	This study
CJD560	GM37(pGB636)/Lac$^-$	This study
CJD561	BRE2076(pGB636)/Lac$^+$	This study
CJD565	294(pGB619)/Lac$^-$	This study
CJD566	294*recA*(pGB619)/Lac$^-$	This study
CJD567	294 *osmZ205*::Tn*10*	This study
CJD571	GM37(pGB637)	This study
CJD572	BRE2076(pGB637)	This study
CJD573	DH1(pGB619)/Ara$^-$ Lac$^-$ (independent isolate)	This study
DH1	*gyrA96 recA1 supE44 hsdR17 endA1 thi-1 relA1*	22
GM37	MC4100 Φ(*proU-lacZ*)hyb2(λ*plac* Mu15)	35
GM230	GM37 *osmZ205*::Tn*10*	24
MC4100	*araD139* Δ(*argF-lac*)*U196 rpsL150 relA1 deoC1 ptsF25 rbsR flbB5301*	8
Phage		
P1*cml*	P1::Tn*9clr*100	47
Plasmids[a]		
pACYC184	P15A replicon; Cm^r Tc^r	10
pDIA100	ColE1 replicon; pBR322*cya*$^+$	41
pGB619	pACYC184*osmZ*$^+$ Tc^r	This study
pGB634	pGB619Δ*hns* Tc^r	This study
pGB636	pGB619Δ(*hns* region II) region III$^+$	This study
pGB637	pGB634Δ(region III) region II$^+$	This study

[a] Details of construction are described in Materials and Methods.

describe experiments in which the veracity of the current model which regards *osmZ* as consisting solely of the gene coding for HNS was tested.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophage. The bacterial strains, plasmids, and bacteriophage used in this study are listed in Table 1.

Growth media, chemicals, and culture conditions. Cells were grown routinely in LB medium (40) or in the defined medium MMA supplemented with 0.4% glycerol as the carbon source (36). MacConkey and tetrazolium indicator plates were supplemented with either arabinose or lactose at 1% (wt/vol) (36). Colonies on indicator plates were scored phenotypically following 18 h of incubation at 37°C. Chemicals were purchased from BDH. Antibiotics were purchased from Sigma and used at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; phosphomycin, 30 μg/ml; and tetracycline, 20 μg/ml. Solid media contained 1.5% agar.

Enzyme assays. Cells to be assayed for β-galactosidase activity were grown to the mid-exponential growth phase, and 100-μl aliquots were permeabilized with sodium dodecyl sulfate-chloroform in 900 μl of Z buffer (36). Enzyme activity was assessed by using the substrate *o*-nitrophenyl-β-D-galactoside (Sigma), and activity was expressed in Miller units (36). In each case, duplicate cultures were each assayed in duplicate and activity was expressed as the mean of the four determinations. Standard deviations were always less than 10%.

Genetic techniques. Genetic marker transductions and genetic mapping were carried out by using temperature-sensitive lysogens of bacteriophage P1*cml* (Table 1) by published methods (47). The presumptive *cya* mutation in CJD512 was shown to be linked to the nearby *ilv* locus by transducing the *ilv-691*::Tn*10* strain BW6159 to Ilv$^+$ with a P1*cml* lysate of strain CJD512 and screening for tetracycline sensitivity on LB medium and for an Ara$^-$ Lac$^-$ phenotype on tetrazolium indicator plates.

Molecular genetic techniques. Bacteriophage lambda DNA was prepared by a small-scale method as previously described (43). Plasmid DNA was prepared as described by Sambrook et al. (43). Transformation experiments with plasmid DNA were carried out by using cells made competent by the $CaCl_2$ or the $RbCl_2$ method (22, 43). pACYC184 recombinant plasmids with foreign DNA insertions in *cat* were cured by transforming the cell with the cloning vector

pACYC184 and selecting for chloramphenicol resistance (Cm^r). Transformants were grown under chloramphenicol selection with repeated subculturing for 50 generations. Since the recombinant plasmids were pACYC184 derived and lacked active *cat*, selecting for Cm^r ensured maintenance of pACYC184 while plasmid incompatibility led to the loss of the recombinant. Loss of recombinant plasmids was confirmed by physical analysis. Restriction endonuclease and DNA ligase enzymes were purchased from Amersham International and used as specified by the manufacturer.

Construction of plasmid pGB619. A 6.4-kb *Eco*RI fragment carrying *hns*, the gene coding for HNS, was identified by inspection of the published physical map of the *E. coli* chromosome and previously published genetic mapping data for *hns*/*osmZ*, together with information on the structure of the chromosome in the vicinity of the locus (20, 24, 28, 34). The *hns* gene was then subcloned as part of this *Eco*RI fragment from recombinant bacteriophage lambda 4D8 from the collection of Kohara et al. (28). The cloned sequence was inserted into the unique *Eco*RI site of plasmid pACYC184 which lies within *cat*, the gene coding for chloramphenicol acetyltransferase (10) (Fig. 1). The ligation mixture was used to transform *E. coli* K-12 strain DH1 (Table 1) to tetracycline resistance, and transformants harboring pACYC184 derivatives with interrupted *cat* genes were detected by plating the transformation mixture on LB plates containing tetracycline and the rosaniline dye crystal violet; *cat*$^+$ cells form deep-purple colonies, and *cat* cells form pale-purple colonies (39). The orientation of the inserts was determined by restriction endonuclease fragment length analysis. Eleven recombinant plasmids in which the direction of transcription of *hns* was oriented against the direction of *cat* transcription were obtained. No recombinant plasmids with the insert in the opposite orientation were detected.

Construction of derivatives of pGB619 lacking the *hns* gene. The *hns* gene was removed from pGB619 by cleaving this plasmid at its unique *Stu*I site, which is close to and downstream from *hns*. Strain DH1 was then transformed with the linear DNA. Tetracycline-resistant transformants were harvested, and plasmid DNA was prepared and screened for molecules which had lost this *Stu*I site and the *Eco*RI site which lies 5′ to the *hns* gene. One plasmid which had undergone this deletion of *hns* in vivo was retained for further analysis and designated pGB634. An inspection of the structure of pGB634 revealed that it had undergone a minimum deletion of cloned chromosomal DNA compatible with the loss of *hns* but not of other cloned DNA sequences (Fig. 2).

Plasmid pGB636 was generated by digesting pGB619 with *Pst*I and religating. All of the *Pst*I sites in pGB619 lie within the insert sequences, and one of these sites is within *hns*. Plasmid pGB636 has lost the two smaller *Pst*I fragments from pGB619, resulting in the inactivation of *hns* due to a deletion of 75% of the 3′ portion of the gene and the loss of a total of 3 kb of cloned chromosomal DNA (Fig. 1 and 2).

Plasmid pGB637 was generated by digesting plasmid pGB634 with *Pvu*II and religating. Plasmid pGB637 has lost the three smaller, contiguous *Pvu*II fragments from pGB634 (a 500-bp vector sequence, a 1.2-kb junction fragment, and 2 kb of cloned DNA), leaving 3.3 kb of cloned chromosomal sequences. Thus pGB634, pGB636, and pGB637 have lost the *hns* gene, pGB634 retains 5 kb of chromosomal DNA immediately downstream of *hns*, pGB636 carries just the 3.1-kb *Pst*I-*Eco*RI fragment of chromosomal DNA distal to *hns*, and pGB637 harbors just the 2.2 kb of chromosomal DNA immediately downstream of *hns* (Fig. 1 and 2).

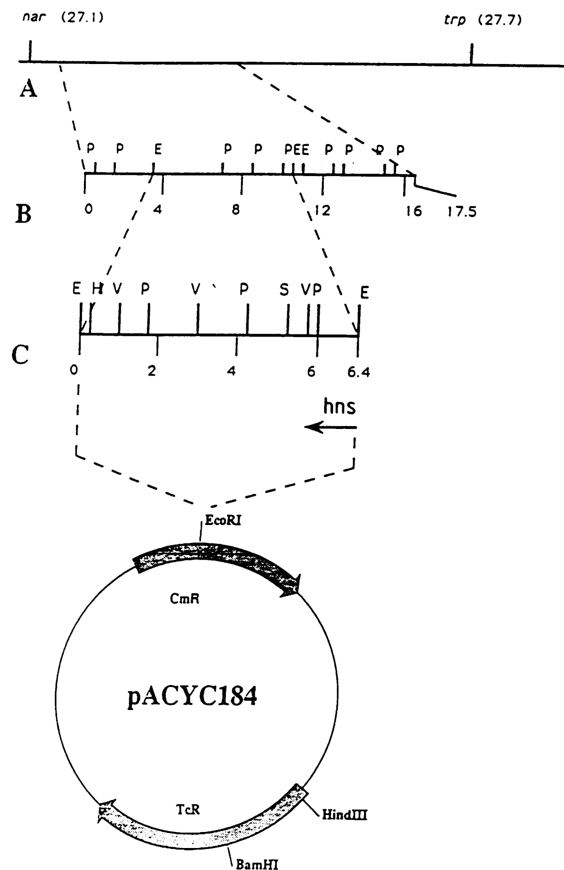

FIG. 1. Cloning the *hns*/*osmZ* locus from *E. coli* K-12 in pACYC 184. (A) The *nar*-to-*trp* region of the *E. coli* chromosome is shown, with map coordinates (in minutes) given in parentheses (2). (B) The 17.5-kb fragment of chromosomal DNA cloned in the recombinant bacteriophage λ derivative 4D8 is illustrated (28). Distances (in kilobases) are indicated below the line. (C) The 6.4-kb *Eco*RI fragment from phage 4D8 subcloned in the *Eco*RI site of plasmid pACYC184 is shown (10). Distances (in kilobases) and the orientation of *hns* (indicated by the arrow) are given below the line. Abbreviations: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Stu*I; V, *Pvu*II; CmR, chloramphenicol resistance; TcR, tetracycline resistance.

RESULTS

Cloning the *hns* gene from *E. coli* K-12 in the multicopy plasmid pACYC184 and complementation of an *osmZ* mutation. To study the effect of increasing the copy number of *hns* on gene expression, we cloned the *hns* gene from *E. coli* K-12 strain W3110 (28) into the multicopy cloning vector pACYC184 (described in Materials and Methods) (Fig. 1). This vector was chosen because it possesses a moderately high copy number of about 20 per chromosome and its P15A replicon is fully compatible with those of the ColE1-based plasmids used elsewhere in the study (10; see below). One recombinant plasmid, pGB619, was retained for further analysis (Fig. 1 and 2). This plasmid could be stably maintained in strain DH1, which harbors the *recA1* mutation, suggesting that a previous report (26) that cloned copies of *hns*/*osmZ* cannot be maintained in *recA* hosts may apply only to particular recombinant plasmids.

Restriction fragment analysis of pGB619 showed that the structure of the insert DNA was identical to that described by others who have cloned the *E. coli hns* gene (data not

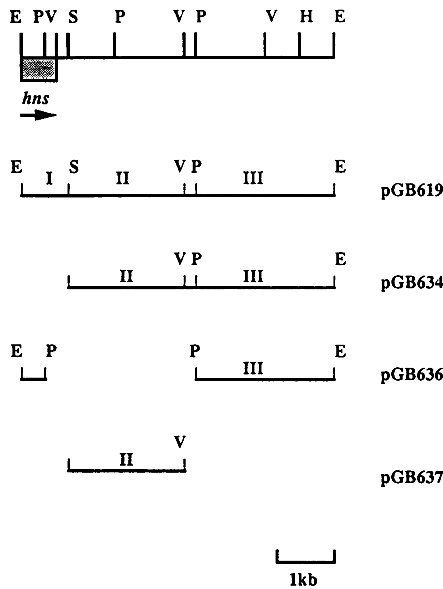

FIG. 2. Location of genetically active regions within *osmZ*. A physical map of the cloned *osmZ* locus is given at the top of the figure. The orientation of the 6.4-kb *Eco*RI fragment is reversed with respect to that shown in Fig. 1. The position of *hns*, the gene coding for HNS, is represented by stippling, and the direction of transcription is indicated by the arrow. In the lower part of the figure, the horizontal lines represent sequences present in the plasmids listed at the right. The genetically active regions are labeled I, II, and III, with region I being synonymous with *hns*. Details of the construction of these plasmids are given in Materials and Methods. Abbreviations: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Stu*I; V, *Pvu*II.

shown). To test pGB619 for *osmZ* activity, we used this plasmid to complement a chromosomal *osmZ* mutation in *E. coli*. Strains of *E. coli* and *S. typhimurium* harboring mutant alleles of *osmZ* are derepressed for transcription of the osmotically inducible *proU* operon, which encodes an uptake system for the osmoprotectant glycine-betaine (12). Strain GM37 harbors a *proU-lacZ* fusion and is wild type for *osmZ* (Table 1). Strain BRE2076 is isogenic with GM37 but carries the *osmZ203* mutation (Table 1). Recombinant plasmid pGB619 was introduced into GM37 and BRE2076 to yield strains CJD542 and CJD547, respectively (Table 1). The effects of pGB619 on the expression of the *proU-lacZ* fusion were assessed by assaying the levels of β-galactosidase in these strains. The results showed that pGB619 complemented the *osmZ203* mutation in BRE2076, reducing the level of *proU-lacZ* expression threefold in cells grown in LB broth (Table 2). These data confirmed that pGB619 did indeed carry an active copy of *E. coli osmZ*. Moreover, the presence of the plasmid caused a significant repression in the level of *proU-lacZ* expression in the *osmZ*$^+$ parent strain GM37 (Table 2). Similar results have been reported by others who have cloned the *E. coli hns/osmZ* locus and are in accord with the view that the *osmZ* copy number is critical and that departure from the normal state of one copy per chromosome can produce unexpected results in complementation experiments (25, 34).

Multicopy *osmZ* appears to repress expression of the arabinose and lactose operons in *E. coli* K-12 strain DH1, but the effect is due to a chromosomal mutation. The *E. coli* K-12 strain DH1 is a commonly used host strain for propagating recombinant plasmids and is wild type for *lac*, the lactose operon (Table 1). Because the complex transcriptional regulation of *lac* includes sensitivity to changes in DNA topology and since the nucleoid-associated protein HNS is known to affect the topology of DNA profoundly, the effect of pGB619 on *lac* expression in DH1 was investigated. Whereas the DH1(pACYC184) strain, CJD543, displayed an approximately 200-fold induction of Lac expression in the presence of the gratuitous inducer isopropyl-β-D-thiogalactopyranoside (IPTG), the DH1(pGB619) strain, CJD512, showed only a 10-fold induction with IPTG (Table 3). To see whether this effect was *lac* specific, we compared strains CJD512, CJD543, and DH1 on MacConkey arabinose indicator plates, since the arabinose operon also shows elements of sensitivity to changes in DNA topology in its regulation. Here, CJD543 and DH1 were found to be clearly Ara$^+$, whereas the pGB619$^+$ strain CJD512 was Ara$^-$ (data not shown). Thus, a DH1 derivative harboring a multicopy plasmid containing active *hns/osmZ* fails to express two carbohydrate utilization operons with known sensitivity to changes in DNA topology.

To discover whether the change in Ara Lac phenotype in the DH1(pGB619) multicopy *osmZ* strain CJD512 was due to the presence of the plasmid, we cured pGB619 from the strain (see Materials and Methods). The cured derivative remained Ara$^-$ Lac$^-$ (data not shown), indicating that it had acquired a mutation not genetically linked to pGB619. To measure the frequency at which Ara$^-$ Lac$^-$ mutants arose in DH1 derivatives harboring the multicopy *osmZ*$^+$ plasmid pGB619, we introduced this plasmid de novo into DH1 by transformation and selection on LB plates containing tetracycline. Transformants were then picked onto tetrazolium indicator plates containing either arabinose or lactose, on which Ara$^+$ or Lac$^+$ strains, respectively, are white and Ara$^-$ or Lac$^-$ strains, respectively, are red. Here, red (i.e.,

TABLE 2. Complementation of *osmZ203* by pGB619

Strain[a]	Relevant genotype	β-Galactosidase activity (Miller units)
GM37	*osmZ*$^+$ Φ(*proU-lacZ*)	296
CJD546	GM37(pACYC184)	340
CJD542	GM37(pGB619)	85
BRE2076	GM37 *osmZ203*	987
CJD548	BRE2076(pACYC184)	1,149
CJD547	BRE2076(pGB619)	324

[a] Cultures to be assayed were grown to mid-log phase in LB medium with aeration.

TABLE 3. Expression of *lac* in CJD512 and complementation of the Lac$^-$ phenotype with a cloned copy of *cya*

Strain	Relevant genotype/phenotype[a]	β-Galactosidase activity (Miller units)[b]: −IPTG	+IPTG
DH1	Wild type	4	750
CJD543	DH1(pACYC184)	4	780
CJD512	DH1(pGB619)/Ara$^-$ Lac$^-$	2	22
CJD573	DH1(pGB619) (independent isolate)/ Ara$^-$ Lac$^-$	1	163
CJD552	CJD512(pDIA100*cya*$^+$)/Ara$^+$ Lac$^+$	5	670

[a] Refers to Ara or Lac phenotype on either arabinose or lactose tetrazolium indicator plates.
[b] Cultures grown to mid-log phase in LB medium.

Ara$^-$ or Lac$^-$) strains arose at a frequency of 2%. Moreover, all Ara$^-$ strains were found to be also Lac$^-$, and vice versa.

The Ara$^-$ Lac$^-$ strain CJD512 carries a *cya* mutation. The *ara* and *lac* operons both require the cyclic AMP (cAMP) receptor protein (CRP) for transcriptional activation (7, 45). Thus, a mutation affecting the ability of the cell to synthesize CRP or cAMP could produce the pleiotropic effect seen in CJD512. An inability to synthesize cAMP could be due to a lesion either at *cya* (coding for adenylate cyclase) or at one of the *pts* loci (coding for members of the phosphoenolpyruvate:carbohydrate phosphotransferase system) (2). Mutations at any of these loci confer resistance to the antibiotic phosphomycin (1, 9, 11), and CJD512 was resistant to phosphomycin at 30 μg/ml, a level inhibitory to DH1. Interestingly, a recent report has shown that *E. coli* K-12 strains deleted for *hns*/*bglY* accumulate mutations in the *ptsHI* operon, making them resistant to phosphomycin and deficient in cAMP (31). To investigate the possibility that CJD512 was cAMP deficient, we grew this strain on MacConkey indicator plates containing either arabinose or lactose, with or without exogenous cAMP. In both cases, cAMP fully restored the ability to transport and utilize the carbohydrate (data not shown). Furthermore, the introduction of pDIA100, a plasmid carrying an active copy of the *E. coli cya* gene, coding for adenylate cyclase (Table 1), also restored the strain CJD512 to Ara$^+$ Lac$^+$ (Table 3). These results showed that the mutation in CJD512 was not in *crp*, the gene coding for CRP. To distinguish between the possibility that CJD512 harbored a mutation in *ptsHI* (as was the case in the work of Lejeune and Danchin [31]) and the possibility that CJD512 lacked active adenylate cyclase, the mutation in CJD512 was genetically mapped by P1*cml* transduction (see Materials and Methods) and shown to be 17% linked to *ilv* at 84.9 min. The *cya* gene maps to 85.7 min on the *E. coli* genetic map, whereas *ptsHI* maps to 52.2 min (2). Thus, the mutation was in *cya* and not in *ptsHI*.

The mutagenic properties of multicopy *osmZ* are seen in both *recA* and *recA*$^+$ backgrounds. The previously described mutagenic effects of *hns*/*bglY* deletions were found to be independent of *recA*, the gene coding for the RecA protein, the central enzyme of the general recombination system (2, 31). *E. coli* K-12 strain DH1 is *recA* and also carries a topoisomerase mutation, *gyrA96*, a mutant allele of the gene coding for the A subunit of DNA gyrase (17, 22) (Table 1). To study the possible contribution of *recA* to the mutagenic effects described above and to eliminate any potential contributions from the poorly characterized *gyrA96* lesion in DH1, the experiments with DH1 were repeated in the *gyr*$^+$ congenic *E. coli* K-12 strains 294 and 294*recA* (Table 1).

The results obtained with 294(pGB619) and 294*recA* (pGB619) were similar to those described above for the *recA* strain DH1(pGB619); i.e., 2% of 294(pGB619) transformants and 2% of 294*recA*(pGB619) transformants were Ara$^-$ Lac$^-$. Since 294 and 294*recA* are *gyr*$^+$, the *gyrA96* lesion in DH1 does not contribute significantly to the acquisition of the Ara$^-$ Lac$^-$ phenotype. To assess the frequency at which the Ara$^-$ Lac$^-$ phenotype was acquired in plasmid-free strains, 294 cultures and 294*recA* cultures were grown to stationary phase in LB medium and then plated for single colonies on lactose tetrazolium indicator plates. In both cases, Ara$^-$ Lac$^-$ mutants were not detected at frequencies above 10^{-9}. Thus, the mutagenic effect is due to pGB619 and occurs at the same frequency in the presence and absence of an active *recA* gene.

The level of *lac* expression in phenotypically Lac$^-$ DH1 (pGB619), Lac$^-$ 294(pGB619), and Lac$^-$ 294*recA*(pGB619) is variable. Other DH1(pGB619) transformants with an Ara$^-$ Lac$^-$ phenotype on tetrazolium indicator plates were found to express *lac* at higher levels than did CJD512. One, CJD573, was found to express *lac* to a level almost eightfold higher than CJD512 upon induction with IPTG, although this was still almost fivefold below the wild-type level seen in DH1 (Table 3). As with CJD512, the reduction in *lac* expression in CJD573 could be reversed by adding exogenous cAMP or by introducing the *cya*$^+$ plasmid, pDIA100 (not shown). These results suggested that the nature of the *cya* mutations induced by pGB619 varied in different Ara$^-$ Lac$^-$ transformants of DH1. However, all mutants were found to be equally resistant to 30 μg of phosphomycin per ml.

TABLE 4. Expression of *lac* in Lac$^-$ derivatives of 294 and 294*recA* harboring pGB619 and in an *osmZ205*::Tn*10* derivative of 294

Strain	Relevant genotype/phenotype[a]	β-Galactosidase activity (Miller units)[b]:	
		−IPTG	+IPTG
294	Wild type/Ara$^+$ Lac$^+$	4	4,020
CJD565	294(pGB619)/Ara$^-$ Lac$^-$	4	1,170
294*recA*	*recA*/Ara$^+$ Lac$^+$	3	3,800
CJD566	294*recA*(pGB619)/Ara$^-$ Lac$^-$	4	600
CJD567	294 *osmZ205*::Tn*10*/Ara$^+$ Lac$^+$	4	3,176

[a] Refers to Ara or Lac phenotype on either arabinoase or lactose tetrazolium indicator plates.

[b] Cultures were grown to mid-log phase in MMA plus glycerol.

The same variation in β-galactosidase expression was seen when 294(pGB619) and 294*recA*(pGB619) transformants showing an Ara$^-$ Lac$^-$ phenotype on tetrazolium indicator plates were assayed for β-galactosidase activity. Data for two isolates, CJD565 and CJD566, are shown in Table 4. These became phenotypically Lac$^+$ when the *cya*$^+$ plasmid pDIA100 was introduced by transformation (data not shown). Although each expressed *lac* to a level significantly below that of the Lac$^+$ parent in the β-galactosidase assay, there was still ample evidence of *lac* expression in these phenotypically Lac$^-$ transformants. As with CJD573, the differences in *lac* expression may reflect differences in the nature of the mutations affecting *cya* promoted by pGB619. It was not possible to explain why CJD565 and CJD566, which expressed approximately 1,200 and 600 U of β-galactosidase activity in broth, respectively, displayed a Lac$^-$ phenotype on indicator plates.

Effect of an *osmZ* mutation on expression of *lac* in *E. coli* K-12 strain 294. Given the dramatic effect of a multicopy plasmid harboring the *hns* gene on expression of the cAMP-CRP dependent carbohydrate utilization operons *ara* and *lac*, the effect of a transposon Tn*10* insertional mutation in the coding sequences of the chromosomal *hns*/*osmZ* gene on Ara and Lac expression was assessed. The *osmZ205*::Tn*10* mutation from *E. coli* K-12 strain GM230 (Table 1) was introduced into strain 294 by transduction with bacteriophage P1*cml*. No Ara$^-$ or Lac$^-$ transductants were detected at frequencies above 10^{-9} among tetracycline-resistant transductants on tetrazolium indicator plates. Thus, the effect of the presence of pGB619*osmZ*$^+$ was not repeated in strains deficient in *osmZ*. To investigate quantitatively the effect of the *osmZ205*::Tn*10* mutation on *lac* expression in strain 294, we retained one transductant, CJD567, for further analysis. When assayed, CJD567 expressed a level of β-galactosidase activity only 1.3-fold lower than that of the

TABLE 5. Effect of the Δ*hns* plasmid derivatives of pGB619 on expression of the *proU* operon

Strain	Relevant genotype/phenotype	β-Galactosidase activity (Miller units)[b]:	
		−NaCl	+NaCl
GM37	Wild type/Lac$^-$	165	1,723
BRE2076	GM37 *osmZ203*/Lac$^+$	936	1,741
CJD555	BRE2076(pGB634)/Lac$^+$	4	4
CJD553	GM37(pGB634)/Lac$^+$	3	4
CJD554	GM37(pGB634)/Lac$^-$	50	414
CJD571	GM37(pGB637)/Lac$^-$	349	2,328
CJD572	BRE2076(pGB637)/Lac$^+$	1,939	4,552
CJD559	GM37(pGB636)/Lac$^+$	122	95
CJD561	BRE2076(pGB636)/Lac$^+$	804	771

[a] Refers to the Lac phenotype when grown on standard (i.e., low-osmolarity) MacConkey lactose indicator plates.
[b] Cultures grown to mid-log phase in LB medium with or without 0.3 M NaCl.

parent strain, 294, showing that the effect of the *osmZ205*::Tn*10* mutation on *lac* expression was marginal (Table 4). When *osmZ*$^+$ strains 294 and 294*recA* were compared for *lac* expression, the *recA* mutant was found to express β-galactosidase at levels comparable to those found in strain 294 (Table 4). As expected, expression of the *lac* operon is not influenced by the presence of the *recA1* mutation in strain 294*recA*.

The mutagenic property of plasmid pGB619 involves sequences additional to *hns*, the gene encoding HNS. To assess the contribution of *hns* to the mutagenic effect on *cya* associated with the presence of pGB619 in *E. coli*, this gene was deleted from pGB619 to yield the plasmid pGB634 (see Materials and Methods) (Fig. 2). When this plasmid was introduced into *E. coli* K-12 strains 294 and 294*recA* by transformation, the pGB634$^+$ transformants gave rise to Ara$^-$ Lac$^-$ mutants at a frequency of approximately 10^{-3}, which was 10-fold lower than in cells harboring pGB619. The mutations in the pGB634 transformants which were responsible for the Ara$^-$ Lac$^-$ phenotypes were complemented by a plasmid harboring an active copy of *cya* (data not shown). This suggested that the mutagenic effect of the multicopy cloned DNA sequences was only partly due to *hns* and that a second, neighboring genetic factor was also involved. The *osmZ*$^+$ plasmid pGB619 was capable of complementing a chromosomal *osmZ* mutation. To test the hypothesis that *hns* was synonymous with *osmZ*, the effect of the Δ*hns* plasmid pGB634 on *proU* expression was assessed in an *osmZ* and in an *osmZ*$^+$ genetic background.

The Δ*hns* plasmid pGB634 alters expression of *proU*. The Δ*hns* plasmid pGB634 was introduced to BRE2076 by transformation, and one transformant, CJD555, was retained for further analysis (Table 1). CJD555 was Lac$^+$ on MacConkey lactose plates; however, when it was grown in low-osmolarity medium and tested for *proU-lacZ* expression by β-galactosidase assay, the fusion was found to be almost completely repressed (Table 5). Moreover, when CJD555 was grown at high osmolarity and assayed for β-galactosidase activity, the *proU-lacZ* fusion was not found to be induced (Table 5). Thus, while CJD555 displayed a Lac$^+$ phenotype on MacConkey lactose indicator plates, it constitutively expressed low levels of β-galactosidase activity when grown in broth at either high or low osmolarity.

Given the negative effects of the Δ*hns* plasmid pGB634 on *proU* expression in the *osmZ203* strain BRE2076, its effects in the *osmZ*$^+$ parental strain GM37 were assessed. GM37 (pGB634) transformants were screened on MacConkey lactose indicator plates. Surprisingly, GM37(pGB634) transformants were strongly Lac$^+$ in 72% of cases. One of these, CJD553, was retained for further study and was found to express *proU* at an extremely low level when grown at either high or low osmolarity (Table 5). The remaining 28% of GM37(pGB634) transformants were Lac$^-$ on indicator plates, with 20% producing Lac$^+$ papillae or sectored Lac$^-$-Lac$^+$ colonies. This was in contrast to the situation with BRE2076(pGB634) transformants, which had a uniformly Lac$^+$ phenotype on these indicator plates at a frequency of 100%. One Lac$^-$ GM37(pGB634) transformant (i.e., one having a phenotype similar to that of the parent strain, GM37, when grown on MacConkey lactose indicator plates), was retained for further analysis and designated CJD554 (Table 1). When assayed for β-galactosidase activity at low and high osmolarity, CJD554 displayed an overall reduction in osmotically inducible *proU* expression when compared with GM37 (Table 5). This indicated that plasmid pGB634 exerted a negative effect on *proU* expression in this strain.

These data suggested that the fragment of chromosomal DNA retained in the Δ*hns* plasmid pGB634 had a major effect on the expression of the *osmZ*-dependent *proU* operon. Consequently, regarding the *osmZ* locus as being equivalent to the gene coding for the nucleoid-associated protein HNS is probably an oversimplification.

Detection of additional genetically active regions within the *osmZ* locus. To find the DNA sequence common to pGB619 and pGB634 responsible for the aberrant expression of *proU* in CJD553, CJD554, and CJD555, deletions of the chromosomal DNA in these plasmids were made in vitro and the resulting molecules were introduced into the *proU-lacZ* fusion strain GM37 and its *osmZ* derivative BRE2076 by transformation. The construction of pGB619 and pGB634 plasmid derivatives pGB636 and pGB637 is described in Materials and Methods, and the structures are shown in Fig. 2. The effects of these plasmids on *proU-lacZ* expression at high and low osmolarity in *osmZ*$^+$ and *osmZ203* backgrounds are summarized in Table 5.

The results obtained indicate that the 6.4-kb fragment of chromosomal DNA harbored by pGB619 includes at least three genetically active regions, I, II, and III (Fig. 2). Region I is synonymous with *hns*. Region II lies within the *hns*-proximal sequences, and, when carried in plasmid pGB637 (i.e., in the absence of regions I and III) and introduced into GM37 (to yield CJD571) and into BRE22076 (to yield CJD572), it had a strongly positive influence on the expression of *proU* (Table 5). Region III lies within the sequences most distal to *hns*, and, when carried in plasmid pGB636 (i.e., in the absence of regions I and II), it had the effect of maintaining *proU* expression at a constitutive level in the presence or absence of osmotic induction when harbored by GM37 (CJD559) or BRE2076 (CJD561) (Table 5). Combining regions II and III in plasmid pGB634 in the absence of region I (i.e., the *hns* gene) resulted in an overall negative effect on the expression of *proU* in both GM37 (CJD553 and CJD554) and BRE2076 (CJD555) (Table 5). These data suggested that the 6.4 kb of cloned chromosomal DNA in plasmid pGB619 contained a complicated regulatory system whose components interacted with each other genetically and that, when acting collectively or individually, they altered the expression of the *proU* operon.

Mutagenic effects associated with the Δ*hns* plasmids in the *osmZ*$^+$ strain GM37; implications for the Ara and Lac phenotypes of strains 294 and 294*recA*. The introduction of plasmid

pGB634 (harboring regions II and III) or pGB636 (harboring region III) into GM37 gave rise to transformants with a Lac$^+$ phenotype on MacConkey lactose indicator plates (as described above). Furthermore, all of these Lac$^+$ transformants were found to have become sensitive to kanamycin. Conversely, when plasmid pGB637 (harboring region II) was introduced into GM37, 100% of the tetracycline-resistant transformants retained their kanamycin resistance marker and were Lac$^-$ on MacConkey lactose plates.

Thus, the acquisition of a Lac$^+$ phenotype at low osmolarity and the loss of kanamycin resistance are associated with the possession of region III in multicopy. Possessing region II alone in multicopy did not lead to these unusual phenotypes, and possession of both regions II and III in multicopy led to a mixture of the Lac$^+$ Km^s and the Lac$^-$ Km^r phenotypes at a ratio of approximately 3:1, as was seen when pGB634 was introduced into GM37. This confirmed the impression that the activities of regions II and III are in contention in the cell.

In GM37 and its derivatives, kanamycin resistance is specified by the gene coding for a type II neomycin phosphotransferase which is an integral component of the λp*lac*Mu insertion in the *proU* operon (Table 1). In strains which had lost resistance to kanamycin, the resistance marker could not be rescued by transduction with bacteriophage P1*cml*. This failure in marker rescue was not due to an inability on the part of P1*cml* to form lysogens or lysates on these strains. In strains which retained resistance to kanamycin, this marker could be rescued at high efficiency by P1*cml* transduction. These results suggest that the aberrant phenotypes in the pGB634$^+$ Lac$^+$ and the pGB636$^+$ Lac$^+$ strains were due to a linked mutation rather than to repression by a plasmid-encoded *trans*-acting regulatory factor.

Since these chromosomal DNA sequences had previously been shown to exert mutagenic effects at *cya* and hence alter the expression of the *ara* and *lac* operons, the effects of plasmids harboring DNA from regions II and III on expression of the *ara* and *lac* operons were assessed. Whereas pGB634 (harboring regions II and III) gave rise to Ara$^-$ Lac$^-$ transformants of strain 294 or 294*recA* at a frequency of about 10^{-3}, plasmids harboring these regions singly did not give rise to Ara$^-$ Lac$^-$ transformants of 294 or 294*recA* at frequencies greater than 10^{-9}. Thus, while regions II and III have significantly different effects on the expression of the *proU* operon, they exert only negative effects on *ara* and *lac* expression (via *cya*) when present together on a multicopy plasmid.

DISCUSSION

Although mutations in the *osmZ* locus were isolated originally as a result of their effects on expression of the osmotically inducible *proU* operons in *E. coli* and *S. typhimurium* (24), it is now known that *osmZ* is allelic with several regulatory mutations affecting the expression of unlinked genes and controlling the kinetics of at least one site-specific recombination system (reviewed in reference 25). All of these mutations have been shown to map to the gene coding for the nucleoid-associated protein HNS. For this reason it is proposed that this gene should be named *hns*, as suggested originally by Pon et al. (38). Now that the location of the gene has been correctly determined, the adoption of a uniform nomenclature should reduce the scope for confusion in studies in this field.

The experiments described in this paper indicate that in terms of its effects on *proU*, the *osmZ* locus is composed of at least three genetically active regions, here designated regions I, II, and III, with region I being synonymous with *hns*. Each alters *proU* expression, and combinations consisting either of I, II, and III or of II and III alter expression of the *E. coli ara* and *lac* operons, specifically through the promotion of mutations affecting *cya*, the structural gene coding for adenylate cyclase. The effect of regions II and III on *hns*-dependent systems other than *proU* has not been formally addressed. The published structures of *hns* clones suggest that in most cases, regions II and III have not been included (20, 26, 34). These regions were almost certainly cloned by Kawula and Orndorff (27) as part of their molecular analysis of *hns/pilG*. In this case a plasmid harboring sequences approximating to region II and harboring an insertional mutation in *hns* did not alter the expression of the fimbrial operon in an *hns/pilG* mutant (27). Thus, the effects of regions II and III may vary from one *hns*-dependent system to another.

The plasmids described in this study caused mutations affecting expression of the *cya* locus and expression of kanamycin resistance. Mutator activity has been associated with the *hns* locus previously. Deletions of *hns/bglY* have been reported to be mutagenic both for chromosomal markers and for plasmid DNA and include deletions at *ptsI-cysK*, which affect the ability of the cell to synthesize cAMP (31). Results from the present work show that mutagenic properties are associated with cloned copies of the *osmZ* locus and that a plasmid carrying regions I, II, and III also alters the expression of cAMP-dependent systems, although this is not through mutations at *pts* but via effects at *cya*, the gene coding for adenylate cyclase (2). A plasmid carrying regions II and III exerts a similar effect but at a lower frequency. Like the mutagenic effects associated with the *hns/bglY* deletions, these are *recA* independent. However, the frequencies for mutator effects associated with *osmZ* sequences in multicopy are approximately 10^3- to 10^4-fold higher than those due to the *hns/bglY* chromosomal deletions (31). Why a deletion at *hns* should promote *ptsI* mutations while a multicopy plasmid harboring the same gene and flanking sequences yields *cya* mutations is unknown.

Mutator effects associated with region III are particularly dramatic in the case of expression of the kanamycin resistance gene associated with the *proU-lacZ* fusion in *E. coli*, with strains harboring multicopy plasmids which include region III losing (at a frequency of 72%) the ability to grow on LB plates containing 50 μg of kanamycin per ml. Intriguingly, deletions in a gene called *irk*, which has been mapped close to *hns*, permit *E. coli* to grow in the presence of otherwise toxic levels of kanamycin (31). To investigate the possibility that *irk* and region III are allelic, the structures of the *osmZ*$^+$ recombinant plasmids generated in this study were compared with the map of the *E. coli* chromosome in the vicinity of *hns/bglY* and *irk*. Mapping data place *irk* between *hns/bglY* and *galU* (31), while region III lies between *hns* and *nar* (Fig. 1 and 2). However, *hns* is known to be transcribed from a promoter lying at the *galU*-proximal end of the gene, and the direction of transcription is toward *nar* (20, 34). An analysis of the restriction endonuclease mapping data of Lejeune and Danchin for *hns/bglY* suggests that these authors have misaligned this gene with its flanking chromosomal sequences and that if it were expressed according to their scheme, it would be transcribed towards *galU* (31). Thus, it is possible that the *irk* gene was mismapped and could lie to the other side of *hns*, i.e., where region III is located. Alternatively, if the *hns/bglY* gene has simply been misoriented on the *E. coli* chromosome but the

map position of *irk* with respect to neighboring genes is correct, *irk* and region III are distinct.

The complex nature of the *osmZ* locus is exemplified by the observation that different alleles can exert different effects on the same system (16, 25, 27). Furthermore, sensitivity to changes in DNA supercoiling does not necessarily imply dependency on *hns/osmZ*. For example, in this study the *osmZ205*::Tn*10* mutation which has been shown previously to alter DNA supercoiling levels in *E. coli* did not significantly alter expression of the DNA supercoiling-sensitive *lac* operon. In addition, the effect of *hns/osmZ* mutations on the level of negative supercoiling of reporter plasmids varies and is allele and species dependent (16, 25–27). These findings are analogous to reports that some *gyrB* mutants of *E. coli* K-12 are not affected in terms of DNA supercoiling, even though the primary role of DNA gyrase is to introduce negative supercoiling into bacterial DNA, whereas other alleles have dramatic effects of DNA supercoiling (17, 18, 42). Thus, some alleles of *hns/osmZ* may exert effects on cellular processes without significantly perturbing DNA supercoiling in reporter plasmids, while other alleles exhibit both phenomena. The correlation between mutations in *hns* and effects on DNA supercoiling is complex and may involve genes which map close to *hns*, perhaps in the genetically defined regions II and III. Interestingly, an *hns/vir*::Tn*10* mutation in *S. flexneri* which alters expression of a plasmid-linked invasion gene and produces a moderate change in DNA supercoiling is phenotypically suppressed at high frequency by a second-site mutation which maps near the Tn*10* in *hns/virR*. Moreover, this second-site mutation has a dramatic effect on the supercoiling of reporter plasmid DNA (16). Experiments are in progress to establish the relationship of such second-site mutations to the newly discovered genetically active regions described in this work.

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