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Identification of an *Escherichia coli* Gene Homologous to *virR*, a Regulator of *Shigella* Virulence

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Virulence in *Shigella* spp., as well as in strains of enteroinvasive *Escherichia coli*, is regulated by growth temperature. Previously, *virR* had been identified as the gene controlling the temperature-regulated expression of *Shigella* virulence. Since *Shigella* spp. and *E. coli* are also known to share >90% DNA sequence homology, we sought to determine if nonpathogenic *E. coli* K-12 C600 contains a gene homologous to the *Shigella flexneri* 2a gene *virR*. Through the use of transduction and molecular cloning of strain C600 chromosomal DNA we have shown that *E. coli* K-12 does indeed contain a gene functionally homologous to the *virR* of *S. flexneri*.

Shigella spp. are human enteric pathogens whose pathogenicity is characterized by their ability to penetrate (invade) and replicate within the colonic epithelium. It has been demonstrated that *Shigella* virulence is regulated by growth temperature since *Shigella* spp., which are phenotypically virulent when cultured at 37°C, become phenotypically avirulent when cultured at 30°C (16). Enteroinvasive strains of *Escherichia coli*, which elicit a disease with pathogenic properties similar to those produced by *Shigella* spp., have also been shown to be temperature regulated for virulence (25). Recently, a gene responsible for the temperature-dependent regulation of virulence in *Shigella* spp. has been identified and shown to map to the region of the *Shigella* chromosome between *galU* and the *trp* operon (17). This gene, referred to as *virR* for virulence regulator, has been cloned and shown to regulate the expression of *Shigella* virulence in a temperature-dependent manner (17). Moreover, temperature-regulated expression of β-galactosidase from a *lac* operon fusion to a virulence gene promoter was demonstrated with the cloned *virR* (17).

A great deal of evidence exists supporting the close genetic relatedness between *Shigella* spp. and *E. coli*. Heteroduplex analysis has demonstrated an overall DNA homology of approximately 90% (4). Comparisons of structural gene nucleotide sequence (3, 5), electrophoretic analysis of enzyme polymorphism (22), and serotyping and biotyping (12) all strongly point to a close genetic affinity between these two organisms.

Since enteroinvasive strains of *E. coli* are temperature regulated for virulence and *Shigella* spp. and *E. coli* are closely related genetically, we sought to determine whether a laboratory strain of *E. coli* K-12 carries a gene homologous in function to the *virR* gene of *Shigella flexneri*.

Identification of a *virR* homolog by transduction. Our strategy for identifying a *virR* homolog in *E. coli* K-12 was based on previous studies of *virR* in *S. flexneri* which mapped the gene to a chromosomal locus between *galU* and *trp* (17). A lysate of the generalized transducing bacteriophage P1 *vir* was prepared on *E. coli* K-12 C600 (F^- *thi-1 thr-1 leuB6 lacY1 tonA53 supE44* λ^- [15]) by the method of Silhavy et al. (24). The lysate was used to transduce chromosomal DNA adjacent to the *E. coli trp* gene into *S. flexneri* BS214. This strain is deleted for the region spanning *virR-trp* and consequently is constitutive for expression of the invasive phenotype. Trp^+ transductants were selected on M9 plates (8) supplemented with 10 μg of nicotinic acid per ml (Sigma Chemical Co., St. Louis, Mo.). Ten Trp^+ transductants were chosen at random to score for temperature regulation of the invasive phenotype in the HeLa cell invasion assay (11). All 10 Trp^+ transductants were invasive only after growth at 37°C, which indicated restoration of the wild-type phenotype. The degree of invasion for the transductants, measured as a percentage of total HeLa cells counted, was between 50 to 94%, comparable to that obtained for the wild-type strain 2457T at 37°C (approximately 80%). The results observed for the Trp^+ transductants contrast with the results obtained with the parent *virR* mutant, BS214, which is invasive at both 37 and 30°C. Transduction of BS214 with a lysate made on *E. coli* K-12 MG1655 (F^- λ^- [1]) yielded identical results in the HeLa cell invasion assay as those obtained when Trp^+ transductants were compared to the control strains 2457T and BS214. MG1655, also a nonpathogenic *E. coli* K-12 strain, represents a lineage derived from wild-type *E. coli* K-12, which is distinct from that of strain C600. The results obtained for the two separate transductions strongly suggest that a region of the *E. coli* K-12 chromosome corresponding to the region spanning *virR-trp* of the *S. flexneri* chromosome contains a gene(s) homologous in function to *virR* of *S. flexneri*.

Cloning of the *virR* homolog from strain C600. We attempted to clone the *E. coli virR* gene by again taking advantage of the known chromosomal location of *virR* in *S. flexneri*, specifically, the tight linkage between *galU* and *virR* (17). A strain C600 chromosomal library was constructed following the procedure described by Maniatis et al. (15). All restriction and DNA modification enzymes were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. High-molecular-weight C600 chromosomal DNA was isolated and partially digested with *Sau*3A. Partially digested DNA was fractionated on a 10 to 30% continuous sucrose gradient to enrich for fragments in the size range of 20 to 30 kilobases. These fragments were then ligated by using T4 DNA ligase in the cosmid cloning vector pCVD301 (7), which had been digested with *Bam*HI and treated with calf intestinal alkaline phosphatase. Cloned DNA from the C600 cosmid library was packaged in vitro into bacteriophage λ heads by using the Gigapack in vitro packaging kit (Stratagene Cloning Systems, San Diego, Calif.). The cloned DNA was then transduced into *E. coli* ATM016 (F^- *lacY1 glnV tyrT ΔgalU trp srl recA56 metB1 hsdR514 trpR55* λ^- [17]),

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TABLE 1. Regulation of *inv::lacZ* operon fusion in BS255 by pCVD301 GalU$^+$ cosmid clone

Strain	Relevant characteristics	U of β-galactosidase[a]		Ratio (37°C/30°C)
		37°C	30°C	
BS183	*inv::lacZ* VirR$^+$	220	5	44
BS255	*inv::lacZ* VirR$^-$	221	122	1.8
BS255 pCVD301	Cloning vector	270	162	1.6
BS255 pAEH001	GalU$^+$ cosmid clone	47	3	15

[a] Units of β-galactosidase are those described by Miller (18).

which is deleted for *galU*. GalU$^+$ clones were selected by their ability to utilize galactose as a sole carbon source. In three separate transductions, 13 GalU$^+$ clones were obtained. Recombinant plasmid DNA from one such GalU$^+$ clone (pAEH001) was prepared by the method of Birnboim and Doly (2) and purified by cesium chloride-ethidium bromide density gradient centrifugation to be used for further study.

Plasmid clone pAEH001 was used to transform *S. flexneri* BS255 as previously described (6) to determine whether the cloned DNA could complement the *virR* defect in this strain. Strain BS255 is a *virR* deletion derivative of BS183 which contains a *lacZ* operon fusion to a *Shigella* invasion (*inv*) gene, placing the *lacZ* gene under the transcriptional control of a temperature-regulated *inv* promoter (17). This *virR* mutant therefore produces β-galactosidase constitutively at 37 and 30°C. A pAEH001 transformant of strain BS255 was randomly picked and tested to compare its level of β-galactosidase at 37 and 30°C with that of the *virR* deletion mutant BS255 and the original *inv::lacZ* fusion mutant BS183. β-galactosidase activity was measured by the method of Miller (18). The results of this experiment are presented in Table 1. The transformant expressed reduced levels of β-galactosidase at 30°C, and this resulted in a 37 to 30°C β-galactosidase ratio of 15. This result would be expected if the clone harbored a *virR* homolog, and it was not due to the presence of the cloning vector, which alone had no effect on β-galactosidase expression in BS255. The 37 to 30°C ratio, although not as high as that of the parental fusion mutant BS183, is considerably higher than that of the *virR* mutant BS255, which had a 37 to 30°C ratio of <2. Based on the complementation of the *virR* defect and the resultant temperature regulation of the *inv::lacZ* operon fusion in BS255, we are able to conclude that the pAEH001 clone contains a gene(s) homologous to *virR*.

The data for pAEH001, although demonstrating that the clone codes for the *virR* homolog, deviate somewhat from the expected results. *virR* in *Shigella* spp. acts as a transcriptional repressor at 30°C (17) and therefore should repress the operon fusion promoter only at 30°C. The β-galactosidase level for the pAEH001 transformant at 37°C, however, is considerably lower than that observed for the parental strain BS255 at 37°C. This indicates the presence of some repressor activity in the transformant at 37°C. The disparity between the reported results for the *virR* homolog clone and the expected activity of *virR* (BS183 in Table 1) may be explained by the possibility that there is a difference in the activity of the *virR* from *E. coli* K-12. Another more likely explanation is the fact that *virR*, which normally exists in a single copy on the chromosome, is cloned on a multicopy plasmid. A higher gene dosage of *virR* and higher overall levels of the VirR protein may have an effect on repression of genes regulated by *virR* even at the normally derepressed temperature. A similar result was obtained for the cloned *Shigella virR* which also showed some repressor activity at 37°C when present on a multicopy plasmid (17).

On the basis of the results from transduction of chromosomal regions of *E. coli* K-12 C600 and MG1655, which represent two different lineages derived from wild-type *E. coli* K-12, and the cloning of the *virR* homolog from strain C600, our data clearly demonstrate that *E. coli* K-12 harbors a gene(s) functionally homologous to *virR*, the temperature-dependent virulence gene regulator of *Shigella* spp. We also show that the *virR* homolog is located in the same region of the *E. coli* genome (linked to *galU* and *trp*) that it is in *Shigella* spp. This fact is not surprising, since *E. coli* and *Shigella* spp. share >90% overall DNA sequence homology as shown by heteroduplex analysis (4). Moreover, conjugational gene transfer experiments not only confirm a high degree of DNA homology but indicate a chromosomal gene order which is very similar in the two genera (9, 23). Further support of *Shigella-E. coli* genetic relatedness has been shown in studies comparing nucleotide sequences of structural genes. For example, the *ompA* and *crp* genes of *Shigella* spp. and *E. coli* share 98% sequence homology (3, 5). The degree of sequence homology between the *Shigella* and *E. coli* K-12 *virR* genes, however, has not yet been determined. Due to the high degree of sequence homology between the two genera and the similar chromosomal location of the *virR* genes, Southern hybridization analysis with the cosmid clones as probes would be impractical. Subcloning of either gene on a smaller fragment (<30 kilobases) could still result in cross-hybridization of flanking DNA sequences. Although demonstrating sequence homology between the two *virR* genes would provide for interesting speculation on their origins, it remains clear that a *virR* gene(s) carried by nonpathogenic *E. coli* K-12 can genetically complement the defective gene in pathogenic *Shigella* spp.

As mentioned previously, enteroinvasive strains of *E. coli*, like *Shigella* spp., are temperature regulated for virulence, and the presence of *virR* in enteroinvasive *E. coli* would therefore not be surprising. However, the fact that the nonpathogenic *E. coli* K-12 strains harbor a gene capable of regulating virulence genes in *Shigella* spp. raises questions of why *E. coli* K-12 would code for such a gene and what the evolutionary basis is for a nonpathogenic bacteria to maintain a regulator of virulence genes. Possible explanations for the presence of a *virR* homolog in *E. coli* K-12 are (i) that the bacteria has lost the genes necessary for expression of a full (*Shigella*-like) virulence phenotype or (ii) that *virR* serves as a global regulator of temperature-regulated genes, and virulence genes acquired by the bacteria have adapted this system to control their own expression.

The argument supporting the possibility that *virR* may be a virulence gene remaining in *E. coli* K-12 after the loss of other virulence genes is not unprecedented if one considers the transcriptional regulatory systems for toxin expression in *Corynebacterium diphtheriae* (20) and *Vibrio cholerae* (19). In *C. diphtheriae*, toxin expression results from lysogenization of the bacteria by a temperate bacteriophage, β, which codes for the toxin gene (10). A model for bacterial regulation of the diphtheria toxin gene has been proposed which hypothesizes that an aporepressor, coded for by the bacteria, acts in the presence of iron to repress diphtheria toxin gene transcription from the phage genome (20). Studies characterizing various mutations in both the phage and *C. diphtheriae* support this model (13, 27). Moreover, it has been demonstrated that cell extracts from nonlysogenic

(toxin$^-$) strains of *C. diphtheriae* specifically inhibit diphtheria toxin gene expression in vitro while having no effect on the expression of other phage-encoded genes (21). Similarly, naturally occurring, nonpathogenic *V. cholerae* strains, which lack the entire cholera toxin gene, still contain DNA sequences homologous to *toxR*, the regulatory gene for cholera toxin expression (19). In both of these systems the regulatory mechanism or the regulatory gene sequences exist in the absence of the virulence genes that they control, suggesting, as in the case of the *virR* homolog, that this virulence gene regulator exists as a fossil gene which has yet to be eliminated by mutation.

That *virR* may serve as a global temperature-responsive repressor of nonvirulence genes is supported by evidence of several temperature-regulated genes in *E. coli* K-12. Genes of the *tra* operon, involved in the conjugative transfer of DNA between bacteria, have been shown to be growth-temperature regulated. Studies of *traT* and *traG*, as well as the genes responsible for F-pilin biosynthesis, demonstrate that expression of their protein products is temperature regulated (26). *envY* regulates the expression of two temperature-regulated major porin proteins, OmpC and OmpF, with no effect, however, on the temperature-dependent expression of another major outer-membrane protein, OmpT (14). Moreover, *envY* does not map to the same region as *virR* nor does the cloned *envY* complement a *virR* defect in *Shigella* spp. (A. T. Maurelli, unpublished data). These results suggest the activity of a temperature-dependent regulator independent of *envY*. Isolation of additional *Shigella lacZ* operon fusion mutants in our laboratory suggests that certain temperature-regulated operon fusion mutants, although controlled by *virR*, still exhibit a fully expressed virulence phenotype (A. E. Hromockyj and A. T. Maurelli, manuscript in preparation). The evidence for the existence of transcriptional regulatory systems in nonpathogenic *C. diphtheriae* and *V. cholerae* suggests that the toxin genes could have been acquired by the bacteria and adapted to an existing system of regulation. This also may be the case for pathogenic strains of *E. coli* which may have evolved from nonpathogenic *E. coli* K-12.

All of this evidence lends credence to the possibility that *virR* could play a role as a global regulator regulating nonvirulence-associated operons and genes in *E. coli* as well as in *Shigella* spp. However, further study will be required to determine the role, if any, of *virR* in the regulation of nonvirulence-associated genes.

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