The *vacB* Gene Required for Virulence in *Shigella flexneri* and *Escherichia coli* Encodes the Exoribonuclease RNase R*

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*vacB*, a gene previously shown to be required for expression of virulence in *Shigella* and enteroinvasive *Escherichia coli*, has been found to encode the 3′–5′ exoribonuclease, RNase R. Thus, cloning of *E. coli* *vacB* led to overexpression of RNase R activity, and partial deletion or interruption of the cloned gene abolished this overexpression. Interruption of the chromosomal copy of *vacB* eliminated endogenous RNase R activity; however, the absence of RNase R by itself had no effect on cell growth. In contrast, cells lacking both RNase R and polynucleotide phosphorylase were found to be inviable. These data indicate that RNase R participates in an essential cell function in addition to its role in virulence. The identification of the *vacB* gene product as RNase R should aid in understanding how the virulence phenotype in enterobacteria is expressed and regulated. On the basis of this information we propose that *vacB* be renamed *rnr*.

Exoribonucleases play an important role in RNA maturation, turnover, and degradation (for reviews, see Refs. 1 and 2). In *Escherichia coli* eight distinct exoribonucleases have been characterized. Most of them display a degree of overlap in their function. For example, six of the eight, including RNases II, D, BN, T, PH, and polynucleotide phosphorylase (PNPase),1 participate in the 3′–matured tRNA precursors (3). Recently, the maturation of the small stable RNAs, M1 RNA, 10Sa RNA/tmlRNA, 6S RNA and 4.5S RNA, was examined and found to involve many of the same exoribonucleases (4). It is also known that strains lacking RNases II, D, BN, T, and PH in combination are inviable, but the presence of any one of the five enzymes is sufficient to confer viability, although with varying degrees of effectiveness (5).

RNase R is one of the eight exoribonucleases. It acts nonspecifically on poly(A), poly(U), and ribosomal RNAs (rRNA) in *vitro* (1, 6–8). The enzyme was initially identified 20 years ago in an *E. coli* strain deficient in RNase II (6). Whereas RNase II accounts for more than 95% of the activity against poly(A) and poly(U) in crude cell extracts, the residual activity against these substrates and rRNA is due primarily to RNase R (1, 5, 7). Based on its gel filtration properties, RNase R is apparently a protein of ~85 kDa (8). However, despite all of this biochemical information, essentially nothing was known about the gene encoding RNase R other than that it mapped to the last quarter of the *E. coli* chromosome.

In this paper we report the identification and characterization of the gene that encodes RNase R and show that it is the *E. coli* *vacB* gene. *vacB* was originally described in *Shigella flexneri* as a chromosomal gene required for expression of the virulence genes carried on the large plasmid of this organism (9). We were led to consider *vacB* as a candidate for the gene encoding RNase R because (a) sequence analysis revealed that it is homologous to the *rnb* gene that encodes another exoribonuclease with similar properties, RNase II (10); (b) the deduced size of the VacB protein (~92 kDa) agreed closely with that known for RNase R; and (c) *vacB* is located at 95 min on the *E. coli* chromosome (9), a position consistent with the earlier mapping studies of the gene encoding RNase R. Based on these considerations, we cloned and characterized the *E. coli* *vacB* gene. The data obtained from these studies demonstrate that the *vacB* gene does indeed encode RNase R, and we propose that it be renamed *rnr*. The identification of VacB as an exoribonuclease has important implications for the understanding of virulence associated with enterobacteria.

**EXPERIMENTAL PROCEDURES**

*Bacterial Strains and Plasmids*—All strains used were *E. coli* K-12 derivatives. Strain C600 was used to amplify λ phage DD947. Strains UT491 (Δ[lac-pro-hsdS (r m ) lacZ]car+car- ) (5) and JM109 (Promega) were used for transformation and plasmid preparations, respectively. Strain CF881 (carB xthA rna) was used for linear transformation, and wild type strain CA265 and two RNase II− strains, CA265II− and CAN20–12E (RNase I F D B N− ) (8), were employed for RNase R assays. Bacteriophage P1vir, used for transduction, was from our laboratory stock.

The *E. coli* genomic clone D DD947, which contains a 20-kb fragment carrying *vacB*, was a gift from Dr. Frederick R. Blattner (University of Wisconsin-Madison) and was used to prepare DNA for subcloning. Plasmid pBS(+) (Stratagene) was used as the cloning vector. Plasmids pUC4K (Pharmacia) and pBR325 provided the Kan′ and Cam′ cassettes used to interrupt *vacB*.

Plasmid pPSV was constructed by subcloning a 4.58-kb EcoRI-NheI fragment from λ DD947 into the EcoRI-XhoI sites of pBB5 (10). To interrupt the *vacB* gene on pPSV, a 1.23-kb PstI fragment was deleted from pBR325, and the remaining fragment was self-ligated to generate plasmid pPSV. The Kan′ cassette, a 1.25-kb PstI fragment from pUC4K, was inserted into the PstI site of pPSV to create plasmid pBSVK. Likewise, plasmid pBSVK was constructed by inserting the 1.23-kb Cam′ cassette from pBR325 into the PstI site of pPSV (see Fig. 1). Plasmid pBSR was constructed by transferring the 2.72-kb XmnI fragment of pBSV into the HindIII site of pBSV(+ ) such that *vacB* was placed under the control of the lac promoter. Because of a stop codon upstream of *vacB*, its gene product is not translationally fused to lacZ. In this plasmid the upstream gene, *yeb*, and most of the downstream gene, *yjh*, are deleted (see Fig. 1).

**Growth Conditions**—Cells were grown either in YT medium containing 0, 0.2, or 0.5% glucose in Tryptone broth supplemented with 0.2% lactose or in M9 medium supplemented with 1 mM thiamine HCl and 0.2% glucose (11). For solid media, 1.5% agar was added. Antibiotics were present at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 25 μg/ml; chloramphenicol, 34 μg/ml.

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**Materials**—Restriction endonucleases, T4 DNA ligase, and Klenow fragment DNA polymerase I were obtained from New England Biolabs. Calf intestine alkaline phosphatase, isopropylthio-β-D-galactoside, and Prime-a-Gene Labeling System were from Promega. Bacterial alkaline phosphatase was from Worthington. T4 polynucleotide kinase was a product of Life Technologies, Inc. [γ-32P]ATP (3,000 Ci/mmol) and [γ-32P]ATP (6,000 Ci/mmol) were from NEN Life Science Products. [3H]Poly(A) was obtained from Amersham Pharmacia Biotech. Poly(A) was from Sigma. [3H]rRNA was kindly provided by Zhihua Zhou and was prepared from [3H]uridine-labeled *E. coli* cells. Ulltrogel AcA44 was from LKB. All other chemicals were reagent grade.

**RESULTS AND DISCUSSION**

**Cloning of the vacB Gene**—To ascertain whether the vacB gene encodes RNase R, it was subcloned from the *E. coli* λ DD947 and collected by centrifugation. Extracts were prepared by sonication of cells suspended in 0.1 volume of a buffer containing 20 mM Tris-chloride, pH 7.5, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 300 mM KC1. The protein concentration of the extract was determined by the Bradford method.

**RNase R Assay**—RNase R activity was measured under optimal conditions by determination of acid-soluble radioactivity released from the substrates, [3H]rRNA or [3H]poly(A), as described (8). Assays were carried out in 50-μl reaction mixtures containing: 20 mM Tris-chloride, pH 8.0, 0.25 mM MgCl₂, 300 mM KC1, 5 μg of [3H]rRNA, or 40 μg of [3H]poly(A). Twenty microliters of extract were added to each reaction, and incubations were carried out at 37 °C for 5–30 min.

**TABLE I**

| Plasmid present | RNase R specific activity against [3H]Poly(A) | [3H]rRNA |
|-----------------|---------------------------------------------|----------|
|                 | nmol/min/mg protein                          | nmol/min/mg protein |
| pBS(+)                  | 38                                           | 6        |
| pBSV                  | 133                                          | 48       |
| pBSVD                 | 48                                           | 7        |
| pBSVK                 | 5                                            | 5        |

**Transformation with the pBS(+) vector served as a control. Inasmuch as RNase II accounts for >95% of the nonspecific exoribonucleolytic activity in an *E. coli* extract (1, 5, 7), it was necessary to use the RNase II background to accurately detect changes in RNase R activity. Transformed cells were grown to an A_{550} ~ 1, and sonicated extracts were prepared and assayed using [3H]poly(A) and [3H]rRNA, substrates of RNase R (7, 8). Under these conditions, activity against poly(A) was elevated 2–3-fold and against rRNA, 5–7-fold when extracts from pBSV-transformed cells were assayed. No elevation of activity was observed for extracts from pBS(+) or pBSVK-transformed cells (data not shown). These initial findings supported the conclusion that one of the three genes present in the insert in pBSV encodes RNase R.**

Because the elevation of RNase R activity was lower than expected for expression from a multicopy plasmid, we examined whether the plasmid may have been lost during growth. In fact, based on plating on YT/ampicillin and on analysis of DNA minipreps, only 10–15% of the cells retained plasmid pBSV; in contrast, all the cells retained pBS(+) . These data suggested that overexpression of at least one of the genes on the pBSV insert is deleterious to cells. Accordingly, we repeated the RNase R assays in cells grown only to an A_{550} ~ 0.3 in an attempt to minimize plasmid loss (Table I). In several experiments, one of which is presented, activity using poly(A) as substrate was elevated 3–5-fold, and with rRNA as substrate, 7–12-fold.

To show that vacB was the gene responsible for the elevated RNase R activity, two additional plasmids, pBSVD, containing a deletion in the vacB gene, and pBSVK, containing both a deletion and a Kan' insertion in vacB (Fig. 1), were also examined for their ability to elevate RNase R activity (Table I). No increase in activity against poly(A) was observed in the presence of these plasmids; moreover, these plasmids were stably maintained in cells. In another experiment to demonstrate that vacB was responsible for the elevation of RNase R activity, an additional plasmid, pBSR, in which the two adjacent genes had been removed (Fig. 1), was also examined. In this plasmid vacB is under the control of the lac promoter. In the presence of 0.2% lactose and 1 mM isopropylthio-β-D-galactoside, up to a 100-fold increase in activity against poly(A) was observed in the CAN20–12E background (data not shown), indicating that the adjacent genes are not needed for elevation of RNase R activity.

To confirm that the elevated RNase R activity actually corresponds to RNase R, samples were analyzed by gel filtration on Ultrogel AcA44. Extracts prepared from CA265II cells transformed with either pBSV or pBS(+) were fractionated, and RNase activity was determined. Assays using [3H]poly(A) as substrate revealed a peak of activity eluting with a molecular weight of ~86,000 from the pBSV extract; however, this peak of activity was non-detectable from extracts of cells transformed with pBS(+) (data not shown). Taken together, these data strongly support the conclusion that vacB encodes RNase R. Inasmuch as the predicted size of the VacB protein is ~92,000, these data also indicate that RNase R is a monomer. Based
Upon the information presented here, we propose that vacB be renamed rnr.

**Interruption of Chromosomal rnr**—The deletion-interruption mutations of rnr present in plasmids pBSVK and pBSVC were introduced into the chromosome of strain CP881 by linear transformation; Kan' Amp' and Cam' Amp' transformants, respectively, were selected for further study. The mutated rnr genes were then transferred to strains CA265 and CAN20–12E by P1-mediated transduction. RNase activity assays (Table II) revealed that the residual activity in strain CAN20–12E, amounting to ~2% of wild type, was decreased even further and rendered undetectable upon introduction of the interrupted rnr genes.

To confirm that the chromosomal rnr gene had, in fact, been substituted with the deletion-interruption mutation, chromosomal DNA from strain CAN20–12E: rnr:kan was subjected to Southern analysis using probes specific for the rnr gene and the Kan' cassette. DNA from the parental strain, CAN20–12E, and from CA265II were used as controls (Fig. 2). When hybridized with the probe specific for rnr, EcoRI-digested chromosomal DNA from all of the strains, including strains CA265II and CAN20–12E that carry wild type rnr and strain CAN20–12E: rnr:kan that contains the Kan' interruption, gave rise to a 9.6-kb band (Fig. 2A, lanes 2–5). This is as expected because the interrupted rnr gene is a deletion-insertion mutant and the fragment deleted is almost identical in size to the Kan' fragment inserted (see “Experimental Procedures”). When hybridized with the probe specific for the Kan' cassette, no band was detected with strains CA265II and CAN20–12E (Fig. 2B, lanes 2 and 3), whereas the same 9.6-kb band was detected with strain CAN20–12E: rnr:kan (Fig. 2B, lanes 4 and 5). These data demonstrate that the rnr gene in the latter strain has been interrupted. Identical results were obtained with the strain containing the Cam' interruption (data not shown).

The isolation of an *E. coli* strain with a null mutation in *rnr* indicates that RNase R is not an essential enzyme for cells cultured in the laboratory even when several other exoribonucleases are absent. To determine whether the absence of RNase R has any effect on cell growth, strain CAN20–12E: rnr:kan was grown on rich medium (YT) and on minimal medium (M9/0.2% glucose) plates at 31, 37, and 42 °C. No growth defect was detected compared with the parental strain, CAN20–12E, under any of these conditions. Moreover, the doubling time of the mutant strain at 37 °C in YT, 0.2% glucose was 30 min, the same as that of the parent. Strain CAN20–12E: rnr:kan also recovered from a 24-h carbon source starvation in M9 salts with the same kinetics as that of the parent, indicating no defect in recovery from starvation. These data suggest that whatever function is served by RNase R, it can be rescued completely by the exoribonucleases that are still present in strain CAN20–12E: rnr:kan. Although this strain lacks RNases II, D, BN, and R, RNases T and PH, PNPase, and oligoribonuclease still remain.

To test whether any of the remaining, known exoribonuclease-
It is likely that rnr is cotranscribed with the adjacent genes, yjeB and yjfH. First, no promoter sequence is evident in the short intergenic sequence between yjeB and rnr or between rnr and yjfH, and second, no transcription terminator is seen downstream of rnr. On the other hand, a possible σ70 promoter, TAGCGA (18 nt) TATCAT, is present upstream of yjeB, and a likely rho-independent terminator, a 7-bp stem followed by 9 U residues, is located 20 nt downstream of the termination codon of yjfH. If these predictions are confirmed, it would indicate that rnr is part of an operon together with the two adjacent genes. Although the identity and functions of these two genes have not yet been established, we have found that yjeB is distantly related to a number of transcriptional repressors and contains a helix-turn-helix motif. yjfH is homologous to a family of RNA methyltransferase genes, including the E. coli spoU (trmH) gene encoding a tRNA 2′-O-methyltransferase (13). Computer analysis also revealed the presence of a REP sequence (14) in the intergenic region between rnr and yjfH.

Based upon its deduced amino acid sequence, RNase R is a basic protein with a pI = 8.78. Whereas basic amino acid residues are distributed throughout the protein, there is a particularly high positive charge density in the C-terminal region. In fact, 40% of the 73 C-terminal residues of RNase R are basic (5 Arg, 24 Lys). Also identified in the C-terminal region is one copy of the S1 RNA binding domain (10). Interestingly, this domain is also present in the C-terminal region of two other E. coli exoribonucleases, RNase II and PNPase (10), both of which have substrate specificities similar to RNase R (1). In addition, as noted earlier, we have now found that RNase R−, PNPase− double mutant strains are inviable, and earlier work had shown that RNase II−, PNPase− strains also do not survive (15). Moreover, E. coli RNase II and RNase R display a high degree of sequence similarity, approaching 60% if conservative amino acid replacements are considered. These observations strongly suggest that RNase II, RNase R, and PNPase may constitute a subfamily within the group of eight E. coli exoribonucleases.

Homologues of E. coli rnr are found in essentially all the sequenced genomes, extending from Mycoplasma to humans (Ref. 16 and this work). Sequence similarities extending over wide regions of these derived proteins range upward from 30% identity and 40% when conservative amino acid replacements are included. These data suggest that the function carried out by RNase R may have been maintained over a wide range of organisms. On the other hand, we have not found homologues of rnr in the sequenced Archaeal genomes. E. coli rnr is clearly orthologous to the vacB gene of S. flexneri (9). However, upon comparing the VacB and RNase R protein sequences, we were surprised to find two interruptions in the near perfect alignment. The first is a 52-amino acid stretch between residues 177 and 228. We found that by inverting a 150-bp EcoRV fragment (bases 1199 to 1349 of GenBank D11024) and introducing two single frameshift corrections near the EcoRV sites, amino acid sequence identity could be restored. Although the inverted vacB segment could be a natural variant because it causes a major disruption within a region conserved among multiple species, we suspect that vacB sequencing errors are the cause of this difference. The second discrepancy, which we also attribute to a likely Shigella vacB sequencing error, is caused by a C-terminal frameshift (a missing G after position 2858 of GenBank D11024). We resequenced this region in E. coli and found perfect agreement with the published E. coli sequence. The reconstructed Shigella VacB and the E. coli RNase R sequences are now 99% identical with only 7 amino acid differences and 29 nucleotide differences. After the C-terminal frameshift reversal, the Shigella vacB sequence extends to the end of GenBank D11024. The last 43 amino acids of the reconstructed Shigella VacB remain unsequenced. The reconstructed partial Shigella VacB protein sequence will appear in the SWISS-PROT data base as a revised entry P30851.

Earlier work showed that the vacB gene product is required for the expression of the virulence phenotype of Shigella and enteroinvasive E. coli (9). A mutation in vacB was found to reduce the expression of several plasmid-encoded virulence antigens, and it was suggested that this deficiency was because of an effect at the postranscriptional level (9). However, the molecular processes affected by the vacB product were not understood. The new information presented here that vacB encodes the 3′→5′ exoribonuclease, RNase R, narrows the possibilities for VacB action in the expression of virulence and should aid in clarifying its role in this process. However, it appears that the function of RNase R extends beyond just affecting virulence. The fact that mutant E. coli K12 strains lacking PNPase and RNase R are inviable suggests that these enzymes carry out an essential function in RNA metabolism that cannot be taken over by any of the other cellular exoribonucleases, even the closely related RNase II. It will be of considerable interest to identify this role as well.

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