JOURNAL OF BACTERIOLOGY, July 1993, p. 4250–4254
0021-9193/93/134250-05$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 175, No. 13

Diversity of Retron Elements in a Population of Rhizobia and Other Gram-Negative Bacteria

SCOTT A. RICE, JEFF BIEBER, JONG-YOON CHUN, GARY STACEY, AND BERT C. LAMPSON*

Department of Microbiology, M409 Walters Life Sciences Building, University of Tennessee, Knoxville, Tennessee 37996-0845

Received 19 January 1993/Accepted 27 April 1993

Genetic elements called retrons reside on the chromosome of *Escherichia coli* and the myxobacteria and represent the first reverse transcriptase-encoding element to be found in a prokaryotic cell. All known retrons produce a functionally obscure RNA-DNA satellite molecule called multicopy single-stranded DNA (msDNA). We report here the presence of msDNA-producing retron elements in a number of new bacterial groups, including strains of the genera *Proteus*, *Klebsiella*, *Salmonella*, *Nannocystis*, *Rhizobium*, and *Bradyrhizobium*. Among a population of 63 rhizobia strains, only 16% contain a retron element. The rhizobia retrons appear to be heterogeneous in nucleotide sequence and show little similarity to previously studied retrons of *E. coli* and the myxobacteria.

A new class of retroelements which encode a reverse transcriptase (RT) structurally similar to the polymerase found in retroviruses has recently been discovered in bacterial cells (8, 11, 16). These genetic elements, termed retrons, are found on the chromosome of several different strains of *Escherichia coli* and the soil bacterium *Myxococcus xanthus* and represent the first known example of an RT-bearing element in a prokaryotic organism (8).

All known retrons produce an unusual RNA-DNA satellite molecule called multicopy single-stranded DNA (msDNA). In *M. xanthus*, msDNA-Mx162 consists of a 162-base single-stranded DNA joined to a 77-base single-stranded RNA (4). However, the DNA strand is, surprisingly, linked to the RNA strand at a specific internal guanosine residue so that a unique 2′-5′ phosphodiester bond (branching out from the 2′ position of this G) joins the RNA to the 5′ end of the DNA strand (see reference 13 for a review of the structure of msDNA). Although msDNAs are produced in abundance (up to 500 copies per cell), their function remains obscure. However, it is now well established that the retron-encoded RT is responsible for the synthesis of msDNA (11, 16).

Presently, retron elements have been reported to exist in only two bacterial groups, *E. coli* and a few members of the myxobacteria, such as *M. xanthus* and *Stigmatella aurantiaca* (3, 6, 7, 15, 19). Here we report the presence of msDNA-producing retron elements in an number of diverse bacterial groups, including the rhizobia, and demonstrate the highly diverse nature of these elements in a natural population of rhizobial strains.

Detection of retron elements in different bacterial groups. Retron elements were discovered by detecting the presence of msDNA by the RT extension method (12, 14). With this method, the DNA portion of msDNA is specifically ^{32}P radiolabeled from a total RNA preparation extracted from each bacterial strain. Twenty or more isolates of *Proteus mirabilis*, *Klebsiella pneumoniae*, *Salmonella* species, rhizobial species, and enterococcal species were screened by this method. Low-molecular-weight bands (Fig. 1) indicate the presence of small labeled DNAs after polyacrylamide gel electrophoresis and autoradiography of the labeling reaction mixes. In addition, half of each labeling reaction mix was also treated with RNase A, causing a shift to a faster-migrating band, indicating that the labeled DNA is also associated with RNA, a hallmark of the msDNA molecule. msDNAs were readily detected in *P. mirabilis* 1174b (Fig. 1A, lanes 9 and 10), *K. pneumoniae* 912b (lanes 3 and 4), *Salmonella* sp. strain SARB-3 (not shown), *Nannocystis exedens* Nael (lanes 7 and 8), and *Rhizobium trifolii* USDA 2065 (lanes 5 and 6).

It is apparent from Fig. 1A that the msDNA from *P. mirabilis* 1174b does not shift to a faster-migrating species after treatment with RNase A. However, other experiments clearly show that RNA is associated with this molecule. For example, treatment of total nucleic acids extracted from *P. mirabilis* with RNase A prior to the RT ^{32}P-labeling reaction produced no radioactive species, indicating that RNA is required for incorporation of label. An atypical structure for this msDNA, in which the 5′ arm of the RNA strand (upstream of the branched rG residue) is only one or two bases long, could explain the failure to detect a shift to a faster-migrating species after digestion of the ^{32}P-labeled form with RNase A.

Four of 23 *P. mirabilis* isolates screened produced msDNA, while only 1 of 21 *K. pneumoniae* isolates and 4 of 70 *Salmonella* isolates screened produced msDNA. However, msDNA was not detected in any of the 30 or so enterococcal strains screened by this method. The *Proteus*, *Klebsiella*, and enterococcal strains were obtained from the clinical bacteriology laboratory of the University of Tennessee College of Veterinary Medicine. The *Salmonella* strains are from the SARB reference collection (2, 18). All rhizobial strains are from the U.S. Department of Agriculture (USDA) Beltsville Rhizobium Culture Collection.

The bacterial genera shown in Fig. 1 to contain msDNA-producing retron elements are representative of three of the four major subdivisions of the purple bacteria (or *Proteobacteria*) (20), including *Proteus*, *Klebsiella*, and *Salmonella* of the gamma subdivision; *Rhizobium* and *Bradyrhizobium* from the alpha subdivision; and *Nannocystis* (a myxobacterium) from the delta subdivision.

All previously characterized retron elements have been found in the bacterial chromosome. Chromosomal DNA was

* Corresponding author.

Downloaded from http://jb.asm.org/ on October 23, 2019 by guest

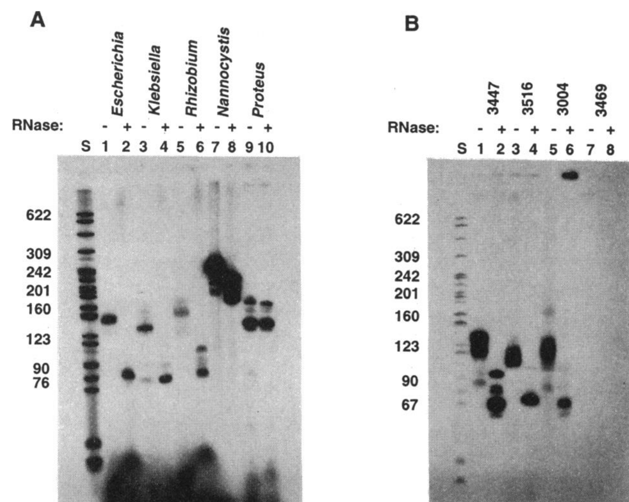

FIG. 1. Detection of ^{32}P-labeled msDNAs by the RT extension method. (A) msDNAs from total RNA prepared from each bacterial strain were specifically labeled with ^{32}P by the RT extension method (12, 14). An aliquot from each labeling reaction mix was treated with RNase A (lanes 2, 4, 6, 8, and 10) prior to electrophoresis to detect a shift to a faster-migrating species, indicating that each labeled DNA is also associated with RNA. msDNAs are clearly present in *K. pneumoniae* 912b (lanes 3 and 4), *R. trifolii* USDA 2065 (lanes 5 and 6), *N. exedens* Nael (lanes 7 and 8), *P. mirabilis* 1174b (lanes 9 and 10), and, as shown previously, *E. coli* CL-1 (lanes 1 and 2). (B) Similarly labeled msDNAs from three additional rhizobial strains. These strains include *Bradyrhizobium* spp. 3447 (lanes 1 and 2), 3516 (lanes 3 and 4), and 3004 (lanes 5 and 6). *R. loti* 3469 appears to lack an msDNA-producing element (lanes 7 and 8). Multiple bands observed in some of the lanes even after RNase treatment may be due to incomplete extension by RT during the labeling reaction, or, alternatively, multiple forms or species of msDNA may exist. Sizes are shown in kilobase pairs.

therefore isolated from each of the msDNA-producing strains described above and subjected to a Southern DNA hybridization experiment with all previously cloned retron genes. Except for *N. exedens*, no cross-hybridization was detected with the four *E. coli* retrons (Ec67, Ec86, Ec73, and Ec107) or the two myxobacterial retrons (Mx65 and Mx162) (data not shown). A strong hybridization signal was detected between an 11-kb *Pst*I chromosomal restriction fragment from *N. exedens* and DNA sequences from the retron Mx162 (not shown). The Mx162 retron was originally discovered and cloned from another myxobacterium, *M. xanthus* (4, 9). Thus, retron elements appear to be widely prevalent, at least among the purple bacteria, occurring in three of the four major subdivisions. In addition, each group appears to have its own unique set of retron elements, with little similarity among these retrons.

Prevalence of msDNAs among a population of rhizobia. The discovery of msDNA in *R. trifolii* (Fig. 1) extends, for the first time, the distribution of retron elements to a new phylogenetic subdivision of the purple bacteria, namely, the alpha subdivision. We were interested therefore in examining the prevalence of these elements among a large population of rhizobia and determining whether any rhizobial retron elements are similar or related to the retrons found in *E. coli* or the myxobacteria. Detection of related retrons in the rhizobia might identify a horizontal transfer event and provide an experimental tool for making evolutionary comparisons between homologous RT genes present in distantly related phyla.

A collection of 63 rhizobial isolates (Table 1) were screened for the presence of msDNA by the RT extension method. This collection (obtained from P. Van Berkum, USDA, Beltsville, Md.) represents isolates obtained at different times, from different legume hosts, and from different geographic locations. Among the 63 isolates, msDNAs were detected in only 10 (16%) (Fig. 1B and Table 1). However, all 10 positive isolates gave strong, clearly labeled bands, with a typical shift to a fast-migrating band after treatment with RNase A, indicating the presence of RNA and DNA in the labeled molecule (Fig. 1B). The 10 retron-encoding rhizobial strains include both fast-growing (*Rhizobium*) and slow-growing (*Bradyrhizobium*) rhizobia. Total DNA from each of eight msDNA-producing strains clearly cross-hybridizes with a *nodYAB* (1.6-kb *Eco*RI fragment) gene probe derived from *Bradyrhizobium japonicum* (1), confirming that these strains are members of the *Rhizobiaceae* (data not shown).

Rhizobial retrons are unique. Southern blots of *Pst*I-digested chromosomal DNAs derived from each of the 10 rhizobial strains which produce msDNAs were prepared. The blots were screened with hybridization probes derived from retron sequences, including the RT gene, from previously cloned elements of *E. coli* and the myxobacteria. However, no cross-hybridization signal was detected under either high- or low-stringency conditions with these probes. Likewise, chromosomal DNA dot blots containing DNA from all 63 strains of the rhizobial collection were also screened with the same hybridization probes. Again, no cross-hybridization signal was detected (not shown).

Rhizobial msDNAs were also used as hybridization probes to identify related retron elements among the rhizobia. The DNA portion of the msDNA molecule was purified from total DNA extracts prepared from seven msDNA-producing rhizobial strains (Fig. 2A). Each purified msDNA was labeled with ^{32}P and hybridized against *Pst*I-digested chromosomes from the other retron-encoding rhizobial strains. For example, when the ^{32}P-labeled msDNA isolated from strain USDA 3242 was used as a hybridization probe against a Southern blot of digested chromosomal DNAs (Fig. 2B and C), a single *Pst*I restriction fragment was detected from the corresponding host cell chromosome (strain 3242) (Fig. 2B, lane 3242). In addition, a single restriction fragment from another strain, USDA 3004, also cross-hybridized with this particular msDNA, indicating that these two strains probably contain the same or a very similar retron element (Fig. 2B, lane 3004). These two strains, 3242 and 3004, appear to be very closely related, having similar restriction fragment length polymorphism profiles and a similar geographic source.

Six other rhizobial msDNAs were similarly isolated and used for hybridization probes. From these hybridizations, the strain pairs 2065 and 2048, 3447 and 3002, and 3516 and 3503 also appear to have a related or closely similar retron element (Table 2). The other remaining msDNAs hybridized to single *Pst*I restriction fragments from their respective host cell chromosomal DNA only (Table 2). Thus, among 10 msDNA-producing rhizobial strains, there appear to be six unique retron elements which show little nucleotide sequence similarity to previously characterized retrons from *E. coli* and the myxobacteria.

Why are retrons so rare? The infrequent occurrence and heterogeneous nature of the retrons observed among this presumably diverse collection of rhizobia are reminiscent of the retrons found in populations of *E. coli*. A survey con-

Downloaded from http://jb.asm.org/ on October 23, 2019 by guest

TABLE 1. Rhizobial isolates[a]

Strain (legume host genus)	USDA strain no.	Geographic source (date)	msDNA produced[b]
Rhizobium sp. (*Acacia*)	3002	Brazil (1959)	+
	3003	Africa (1950)	
	3325	Morocco (1974)	
	3838	? (1976)	+
Bradyrhizobium sp. (*Aeschynomene*)	3516	Florida (1972)	+
	4362		
Bradyrhizobium sp. (*Albizia*)	3004	Maryland (1952)	+
Bradyrhizobium sp. (*Apios*)	3240	Maryland (1939)	
Bradyrhizobium sp. (*Arachis*)	3339	Thailand (1979)	
	3341	Hawaii (1978)	
Rhizobium sp. (*Astragalus*)	3854	Alaska (1962)	
Rhizobium sp. (*Cajanus*)	3472		
Bradyrhizobium sp. (*Canavalia*)	3317	Brazil (1974)	
Rhizobium sp. (*Cicer*)	3378		
	3379	Mexico (1963)	
Bradyrhizobium sp. (*Coronilla*)	3165	Virginia (1935)	
	3167	? (1961)	
Bradyrhizobium sp. (*Crotalria*)	3384	Brazil (1967)	
Bradyrhizobium sp. (*Desmodium*)	3225	Ecuador (1948)	
Bradyrhizobium sp. (*Erythrina*)	3241		
	3242	Maryland (1939)	+
Rhizobium fredii	191	China (1979)	
Rhizobium leguminosarum	2370	Illinois (1933)	
	2429	Hawaii (1978)	
	2435	Holland (1955)	
	2480	Tennessee (1951)	
	2489		
Rhizobium sp. (*Lens*)	2426		
	3404	Colombia (1979)	
Rhizobium loti	3084	Maryland (1946)	
	3468	New Zealand (1961)	+
	3469		
	3471		
	3503		+
	3669	California (1968)	
Bradyrhizobium sp. (*Lotus*)	3074	Minnesota (1954)	
	3470	California (1916)	
Rhizobium sp. (*Lupinas*)	3040	Florida (1940)	
Bradyrhizobium sp. (*Lupinas*)	3045	Florida (1946)	
Bradyrhizobium sp. (*Macrotyloma*)	3451	Zimbabwe (1960)	
Rhizobium medicago	1097	North Dakota (1948)	
Rhizobium meliloti	1011	Maryland (1933)	
	1021a	North Dakota (1948)	
Rhizobium phaseoli	2667	Washington (1948)	
	2669		
	2674	Brazil (?)	
	2676	Colombia (1972)	
	3256	Illinois (1941)	
Rhizobium sp. (*Robinia*)	3436		
Bradyrhizobium sp. (*Stylosanthes*)	3441	Brazil (?)	
	3477	Colombia (1976)	
Rhizobium trifolii	2046	Virginia (1934)	
	2048	Illinois (1934)	+
	2063	Florida (1939)	
	2065	Alabama (1952)	+
	2116	South Carolina (1944)	
	2134	? (1974)	
	2145		
	2156	California (1920)	
Rhizobium sp. (*Trigonella*)	1177	Florida (1939)	
Rhizobium tropici	2744	Brazil (?)	
Bradyrhizobium sp. (*Vigna*)	3447	Thailand (1979)	+
	3456	Wisconsin (1966)	

[a] All strains are from the USDA Beltsville Rhizobium Culture Collection, provided by Peter van Berkum.
[b] As defined by detection of radiolabeled msDNA by the RT extension method.

Downloaded from http://jb.asm.org/ on October 23, 2019 by guest

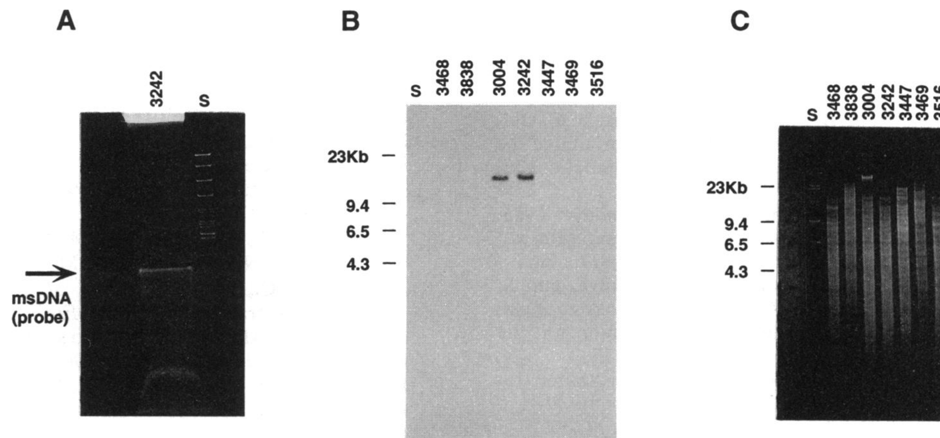

FIG. 2. Southern hybridization with purified msDNA as a probe. (A) Total nucleic acids prepared from *Bradyrhizobium* sp. 3242 was treated with RNase A and electrophoresed on a 5% acrylamide gel, and the satellite DNA band corresponding to msDNA was then excised from the gel and used as a probe labeled by random primer (hexamer) labeling with ^{32}P (5). (B) The msDNA probe was hybridized to a Southern transfer blot of the gel shown in panel C, containing *Pst*I-digested chromosomal DNAs from the rhizobial strains indicated. msDNA from strain 3242 hybridizes to a single *Pst*I chromosomal restriction fragment from host strain 3242, indicating that a single-copy retron element likely resides on this fragment. In addition, the same msDNA also cross-hybridizes to a single *Pst*I restriction fragment from the chromosome of strain 3004, indicating that these two strains probably have similar retron elements encoding similar msDNAs. Lane numbers correspond to USDA strain numbers as listed in Table 1. Lanes designated S contain plasmid pBR322 DNA digested with *Msp*I (A) or lambda DNA digested with *Hin*dIII (B and C) as size standards.

ducted on an *E. coli* reference collection of 72 strains (the ECOR collection) showed that only about 13% of these strains contained a retron (6); of these positive strains, there appear to be four distinct types of retron elements. Likewise, our study indicates that other members of the family *Enterobacteriaceae* appear to have only a few retron-containing strains among the populations sampled. For *P. mirabilis*, only about 17%, for *K. pneumoniae* only 5%, and for *Salmonella* spp. only about 6% of the respective populations screened appeared to contain retron elements.

What is the explanation for the existence of these highly diverse genetic elements in only a few selected strains of the *Rhizobiaceae*? Like *E. coli* populations, selective lineages of rhizobia may have independently acquired or "picked up" a retron element from some unknown source. This transfer of retrons into a few genomes of the rhizobia probably occurred relatively recently, with subsequent horizontal exchange of some retrons occurring between strains of rhizobia as well. Indeed, studies of *E. coli* retrons provide some evidence for this idea. For example, the amino acid codon usage for RT genes found in *E. coli* retrons is clearly different from that of most protein-encoding genes of the host chromosome, indicating that retrons are foreign to *E. coli* (9). Likewise, the lack of nucleotide sequence diversity among several individual (homologous) retrons of the Ec107 type, which are found in several different phylogenetic strains of the ECOR collection, indicates a recent exchange or dissemination of this retron among several *E. coli* strains (10).

Features of the retron elements described here for the rhizobia and previously for *E. coli* are suggestive of a mobile element. However, this remains to be demonstrated directly in the laboratory. If retrons are mobile, perhaps by a retrotransposition mechanism, this appears to be a rare event among natural populations of rhizobia and *E. coli* due to their infrequent occurrence. Contrast this with a survey of the ECOR collection for insertion sequence (IS) elements, which found that almost every strain contains multiple copies of one or more IS elements (17).

As revealed for the rhizobia in this article and previously for *E. coli*, retron elements appear to be a recent guest of the bacterial genome. Where did these retrons come from and what is the evolutionary relationship among the different types of elements? Further study of the prevalence and kinship of retron elements among bacterial groups such as the rhizobia and the myxobacteria may provide some clue as well as clarify questions about the origin of RT and retroelements in general.

TABLE 2. Summary of hybridizations with msDNAs as probes

msDNA probe[a]	Hybridization[b]	Chromosome fragment size[c] (kbp)
3447	3447, 3002	1.1
3838	3838	18.5
3242	3242, 3004	14
2065	2065, 2048	4.3
3516	3516, 3503	8.1
3004	3004, 3242	14
3468	3468	2.6
2048	ND[d]	ND
3002	ND	ND
3503	ND	ND

[a] msDNAs are identified here by the USDA strain number of the bacterial host from which the molecule was originally isolated.
[b] Chromosomal DNAs which hybridized with the indicated msDNA probe are identified by the USDA strain number of the bacterial host.
[c] Size (in kilobase pairs) of the *Pst*I chromosomal restriction fragment which hybridizes with the indicated msDNA probe.
[d] ND, not determined.

We thank Peter Van Berkum (USDA, Beltsville, Md.) for the rhizobia collection, Noel Smith and Robert Selander (Pennsylvania State University) for the *Salmonella* collection, David Bemis (University of Tennessee, Knoxville) for the other enterobacterial strains, and Lawrence Shimkets (University of Georgia, Athens) for *N. exedens* Nael.

Downloaded from http://jb.asm.org/ on October 23, 2019 by guest

REFERENCES

1. **Banfalvi, Z., A. Nieuwkoop, M. Schell, L. Besl, and G. Stacey.** 1988. Regulation of *nod* gene expression in *Bradyrhizobium japonicum*. Mol. Gen. Genet. **214:**420–424.
2. **Beltran, P., S. A. Plock, N. H. Smith, T. S. Whittam, D. C. Old, and R. K. Selander.** 1991. Reference collection of strains of the *Salmonella typhimurium* complex from natural populations. J. Gen. Microbiol. **173:**601–606.
3. **Dhundale, A., T. Furuichi, S. Inouye, and M. Inouye.** 1985. Distribution of multicopy single-stranded DNA among myxobacteria and related species. J. Bacteriol. **164:**914–917.
4. **Dhundale, A., B. Lampson, T. Furuichi, M. Inouye, and S. Inouye.** 1987. Structure of msDNA from *Myxococcus xanthus*: evidence for a long self-annealing RNA precursor for the covalently linked, branched RNA. Cell **51:**1105–1112.
5. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
6. **Herzer, P. J., S. Inouye, and T. S. Whittam.** 1990. Phylogenetic distribution of branched RNA-linked multicopy single-stranded DNA among natural isolates of *Escherichia coli*. J. Bacteriol. **172:**6175–6181.
7. **Hsu, M.-Y., C. Xu, M. Inouye, and S. Inouye.** 1992. Similarity between the *Myxococcus xanthus* and *Stigmatella aurantiaca* reverse transcriptase genes associated with multicopy, single-stranded DNA. J. Bacteriol. **174:**2384–2387.
8. **Inouye, M., and S. Inouye.** 1992. Retrons and multicopy single-stranded DNA. J. Bacteriol. **174:**2419–2424.
9. **Inouye, S., M.-Y. Hsu, E. Eagle, and M. Inouye.** 1989. Reverse transcriptase associated with biosynthesis of the branched RNA-linked msDNA in *Myxococcus xanthus*. Cell **56:**709–717.
10. **Kawaguchi, T., P. J. Herzer, M. Inouye, and S. Inouye.** 1992. Sequence diversity of the 1.3 Kb retron (retron-Ec107) among three distinct phylogenetic groups of *Escherichia coli*. Mol. Microbiol. **6:**355–361.
11. **Lampson, B. C., M. Inouye, and S. Inouye.** 1989. Reverse transcriptase with concomitant ribonuclease H activity in the cell-free synthesis of branched RNA-linked msDNA of *Myxococcus xanthus*. Cell **56:**701–707.
12. **Lampson, B. C., M. Inouye, and S. Inouye.** 1991. Survey of multicopy single-stranded DNAs and reverse transcriptase genes among natural isolates of *Myxococcus xanthus*. J. Bacteriol. **173:**5363–5370.
13. **Lampson, B. C., S. Inouye, and M. Inouye.** 1991. msDNA of bacteria. Prog. Nucleic Acid Res. Mol. Biol. **40:**1–24.
14. **Lampson, B. C., M. Viswanathan, M. Inouye, and S. Inouye.** 1990. Reverse transcriptase from *Escherichia coli* exists as a complex with msDNA and is able to synthesize double-stranded DNA. J. Biol. Chem. **265:**8490–8496.
15. **Lim, D., T. A. T. Gomes, and W. K. Maas.** 1990. Distribution of msDNAs among serotypes of enteropathogenic *Escherichia coli* strains. Mol. Microbiol. **4:**1711–1714.
16. **Lim, D., and W. K. Maas.** 1989. Reverse transcriptase dependent synthesis of a covalently linked, branched DNA-RNA compound in *E. coli* B. Cell **56:**891–904.
17. **Sawyer, S. A., D. E. Dykhuizen, R. F. DuBose, L. Green, T. Mutangadura-Mhlango, D. F. Wolczyk, and D. L. Hartl.** 1987. Distribution and abundance of insertion sequences among natural isolates of *Escherichia coli*. Genetics **115:**51–63.
18. **Selander, R. K., and N. H. Smith.** 1990. Molecular population genetics of *Salmonella*. Rev. Med. Microbiol. **1:**219–228.
19. **Sun, J., P. J. Herzer, M. P. Weinstein, B. C. Lampson, M. Inouye, and S. Inouye.** 1989. Extensive diversity of branched-RNA linked multicopy single-stranded DNAs in clinical strains of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **86:**7208–7212.
20. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.

Downloaded from http://jb.asm.org/ on October 23, 2019 by guest