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Phylogenetic Comparison of Retron Elements among the Myxobacteria: Evidence for Vertical Inheritance

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Twenty-eight myxobacterial strains, representing members from all three subgroups, were screened for the presence of retron elements, which are novel prokaryotic retroelements encoding reverse transcriptase. The presence of retrons was determined by assaying strains for a small satellite DNA produced by reverse transcription called multicopy, single-stranded DNA (msDNA). An msDNA-producing retron appeared to be absent from only one of the strains surveyed. DNA hybridization experiments revealed that retron elements similar to retron Mx162, first identified in *Myxococcus xanthus*, were found only among members of the *Myxococcus* subgroup; that is, each of the seven different genera which constitute this subgroup contained a Mx162 homolog. Another retron element also appeared to have a clustered distribution, being found exclusively within the *Nannocystis* subgroup of the myxobacteria. A retron element of the Mx162 type was cloned from *Melittangium lichenicola*, and its DNA sequence was compared with those of similar elements in *M. xanthus* and *Stigmatella aurantiaca*. Together, the degree of sequence diversity, the codon bias of the reverse transcriptase genes, and the clustered distribution of these retrons suggest a possible evolutionary scenario in which a common ancestor of the *Myxococcus* subgroup may have acquired this retroelement.

In 1984, Yee et al. (39) discovered a small, high-copy-number satellite DNA associated with the gram-negative soil bacterium *Myxococcus xanthus*. This DNA consisted of a single strand of DNA covalently linked by a 2′–5′ phosphodiester bond to an internal guanine residue of a single-stranded RNA (6, 9). These molecules, termed multicopy, single-stranded DNA (msDNA), are encoded by a chromosomal gene called a retron (36). The original retron found in *M. xanthus* is designated Mx162 (16, 39), and a second unique retron element, designated Mx65, resides with Mx162 on the chromosome of *M. xanthus* (5). In addition to coding for the RNA and DNA regions of msDNA, the chromosomal gene also contains an open reading frame (ORF) that shows significant similarity to retroviral reverse transcriptases (RTs) (16). These genes associated with msDNA are the first prokaryotic RTs to be discovered (16, 20, 24). Other types of RT such as the newly discovered intron-encoded ORFs also appear to exist in prokaryotes (8). Synthesis of msDNA is postulated to occur by reverse transcription and is dependent on the production of a functional RT (16, 18, 24). Deletion of the retrons in *M. xanthus* does not affect growth or development of the bacterium (3, 16). Additionally, since the elements are not ubiquitous among bacterial strains, it is apparent that the elements are not required for survival, and despite the widespread nature of retrons, no function has been ascribed to them.

In addition to *M. xanthus* and the myxobacterium *Stigmatella aurantiaca* (9), retrons have been reported in 9 to 15% of all *Escherichia coli* strains tested (11, 20, 23, 35). Recently, we reported the presence of retron elements in several distantly related bacterial groups, suggesting that msDNA and RT genes are prevalent in the prokaryotes (29). As with *E. coli*, only a small percentage (5 to 15%) of strains of *Klebsiella pneumoniae*, *Proteus mirabilis*, *Salmonella* sp., *Rhizobium* sp., and *Bradyrhizobium* sp. tested contain a retron (29).

A number of characteristics distinguish the retron elements of the myxobacteria from retrons found in the rest of the proteobacteria. First, unlike the case for other bacterial groups, in which retrons occur rarely, the elements appear to be nearly ubiquitous among natural *M. xanthus* strains (19). Second, DNA sequence analysis of retrons from *M. xanthus* and *S. aurantiaca* indicates that the codon bias of the RT gene is similar to the codon bias observed for other known myxobacterial genes (16). These features aroused speculation that bacterial RTs of the myxobacteria are ancient elements and that they evolved prior to the emergence of RTs and retroelements in eukaryotes (14). These intriguing evolutionary questions prompted a closer examination of the retroelements of the myxobacteria.

This report presents the findings of an extensive survey of the retron elements found among the myxobacteria. All but one of the 28 strains examined contained an msDNA-producing element. In addition, several different myxobacteria groups were found to contain a retron element similar to Mx162, a retron previously identified in *M. xanthus*. One of the retron elements, from the myxobacterium *Melittangium lichenicola*, was cloned, and its DNA sequence was compared with those of similar elements in *M. xanthus* and *S. aurantiaca*. Together, the degree of sequence diversity, the codon bias of the RT genes, and the distribution of these retrons suggest a possible evolutionary scenario in which a common ancester of the *Myxococcus* subgroup probably acquired this retroelement.

MATERIALS AND METHODS

Bacterial strains and plasmids. The myxobacterial strains used for this study and their sources are listed in Table 1. *E. coli* JM109 and *E. coli* DH5α were used for cloning in conjunction with plasmids pBR322 and pUC19 (38). *M. xanthus* DZF1 was grown at 30°C in CYE broth (28). *Chondromyces* and *Sorangium* strains were grown on VY/2 plates (0.5% baker's yeast, 0.1% $CaCl_2 \cdot 2H_2O$, 1.5% agar) at 30°C (28). All other myxobacteria strains were grown at 30°C on MD1 plates (0.3% Difco Casitone, 0.07% $CaCl_2 \cdot 2H_2O$, 0.2% $MgSO_4 \cdot 7H_2O$, trace elements, 1.5% agar) (28). *E. coli* was grown at 37°C in modified Luria-Bertani medium (25). When necessary, tetracycline (12 μg/ml) or ampicillin (50 μg/ml) was added for selection of plasmids.

Detection of msDNA. Total RNA was purified by the guanidinium thiocyanate method (1) from freshly grown cells or frozen cell pellets. Purified RNA (this fraction also contains msDNA) was stored in ethanol at −80°C. msDNA was

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TABLE 1. Survey of the myxobacteria for retron elements

Organism screened	Strain	msDNA production	Hybridization			Reference	Location and/or strain source
			Mx162	Mx65	Na e434		
Subgroup *Myxococcus*							
Angiococcus disciformis	ATCC 33172	+	+	−	−	This study	
Archangium gephyra	ATCC 25201	+	+	−	−	This study	
Corallococcus (*Myxococcus*) *coralloides*	DK817	+[a]	+[b]	ND[c]	ND	4	Yosemite, Calif.; Dale Kaiser
	ATCC 25202	+	+	−	−	This study	
Cystobacter ferrugineus	Cb fe17	+	+	ND	ND	This study	Hans Reichenbach
	Cb fe18	−	−	−	−	This study	Hans Reichenbach
Cystobacter fuscus	Cb f17	+	+	ND	ND	This study	Hans Reichenbach
Cystobacter violaceus	Cb vi4	+	+	ND	ND	This study	Hans Reichenbach
Melittangium lichenicola	ATCC 25946	+	+	−	−	This study	Hans Reichenbach
Myxococcus fulvus	ATCC 25199	+	+	−	−	This study	
Myxococcus macrosporus	ATCC 29619	+	+	+	−	This study	
Myxococcus stipitatus	ATCC 29611	+	+	−	−	This study	
Myxococcus virescens	ATCC 25203	+	+	+	−	This study	
Myxococcus xanthus	DZF1	+	+	+	−	19	David Zusman
	19 other strains	+	+	+/−	−	19	Hans Reichenbach and Dale Kaiser
Stigmatella aurantiaca	DW4	+	+	−	−	19	Minneapolis, Minn.; David White
Subgroup *Chondromyces*							
Chondromyces apiculatus	Cm a15	+	−	−	−	This study	
	Cm a16	+	−	−	−	This study	
Chondromyces pedicalatus	Cm p5	+	−	−	−	This study	Montes Claros, Brazil; Hans Reichenbach
Sorangium cellulosum	So ce5	+	−	−	−	This study	Cefalù, Sicily; Hans Reichenbach
	So ce7	+	−	−	−	This study	Delphi, Greece; Hans Reichenbach
	So ce11	+	−	−	−	This study	Yucatan, Mexico; Hans Reichenbach
Subgroup *Nannocystis*							
Nannocystis exedens	Na e1	+	−	−	+	This study	Hans Reichenbach
	Na e3	+	−	−	+	This study	Nebraska, Hans Reichenbach
	Na e24	+	−	−	+	This study	Tenerife, Spain; Hans Reichenbach
	Na e30	+	−	−	+	This study	Calpe, Spain; Hans Reichenbach
	Na e39	+	−	−	+	This study	Cefalù, Sicily; Hans Reichenbach
	Na e434	+	−	−	+	This study	Jerusalem, Israel; Hans Reichenbach
	Na e465	+	−	−	+	This study	Hans Reichenbach
	Na e585	+	−	−	+	This study	Pissouri, Cyprus; Hans Reichenbach
	Na e619	+	−	−	+	This study	Mallorca, Spain; Hans Reichenbach
	Na e642	+	−	−	+	This study	Bornholm, Denmark; Hans Reichenbach

[a] Screened by ethidium bromide staining.
[b] Probed by using the *msd* region only for the probe.
[c] ND, not determined.

detected by the RT extension method (19, 21, 29). Labeled products were divided in half, with RNase A treatment of one set of samples, and separated on 4% acrylamide–8 M urea gels. Gels were dried and exposed to X-ray film at −80°C.

Detection of homologous retron elements. Chromosomal DNA was isolated from each myxobacterial strain and digested with restriction endonuclease *Pst*I as described previously (29). Chromosomal DNA digests were separated on 0.7% agarose gels and transferred to nitrocellulose filters. Southern hybridizations were carried out at high stringency (50% formamide, 5× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.7], 5× Denhardt's solution, 0.3% sodium dodecyl sulfate) at 42°C. The Mx162 probe was prepared as a 1.3-kb *Xho*I-*Rsa*I subfragment from plasmid pmsSB7 (29). This fragment contains part of the *msd* gene, which codes for the DNA portion of msDNA, and about two-thirds of the N-terminal region of the RT ORF. The Mx65 probe was prepared as a 1.2-kb *Eco*RI-*Bam*HI fragment from plasmid pBPV-1 (5, 19). msDNA from *Nannocystis exedens* Na e434 was prepared for use as a probe as follows. msDNA was specifically labeled with ^{32}P by the RT extension method, RNase A treated, and then isolated by electrophoresis on a 4% acrylamide–8 M urea gel. The msDNA was cut out of the gel and recovered by elution. Gel-purified restriction fragments were labeled for probes with [α-^{32}P]dCTP by using the random hexamer labeling technique (7) and purified by using Sephadex G-50 columns.

Cloning of a retron homologous to Mx162. *Melittangium lichenicola* chromosomal DNA was digested with *Pst*I and separated by electrophoresis on a 0.7% agarose gel. *Pst*I fragments in the range of 9 to 11 kb were recovered, purified by electroelution, and ligated into the dephosphorylated *Pst*I site of pBR322. Tetracycline-resistant, ampicillin-sensitive colonies were screened by colony hybridization (25), using a 0.75-kb *Eco*RI-*Hin*dIII fragment of retron Mx162 as a probe. This probe hybridized to plasmid pNaeF4.15, containing a 10.8-kb fragment. From this positive clone, a 3.8-kb *Bam*HI fragment was subcloned into a dephosphorylated *Bam*HI site in pUC19 (designated pNaeBB4.0.A4). Overlapping fragments of pNaeBB4.0.A4 were subcloned into pUC19 for sequencing.

DNA sequencing. DNA sequencing of pNaeBB4.0.A4 subclones was performed by the dideoxy-chain termination method of Sanger et al. (30), using a ΔTaq version 2.0 sequencing kit from United States Biochemical. Overlapping clones or synthetic primers were used to ensure that sequencing runs extended through all cloning sites. The DNA sequence was determined for both strands. Single-stranded templates were prepared by alkali denaturation as described in the protocol for the sequencing kit (United States Biochemical). The nucleotide sequence of the DNA portion of the msDNA was determined directly by the method of Maxam and Gilbert (26). msDNA was isolated from freshly grown cells, treated with RNase A, gel purified on a 5% acrylamide gel, and labeled at the 5′ end with [γ-^{32}P]ATP, using T4 polynucleotide kinase (New England Biolabs). Electrophoresis of sequencing reactions was performed by using 6% (wt/vol) acrylamide–8 M urea gels. Sequences were analyzed by using DNA Inspector (version IIe), the GenBank sequence database (version 7.3), and the package of the Genetics Computer Group of the University of Wisconsin (2).

Silent substitutions and codon bias. The degree of nucleotide sequence variation between two homologous genes is the result of the accumulation of nucleotide substitutions over a period of time since the two genes diverged from a common ancestor. For most genes, changes tend to occur at a uniform frequency or in a clock-like fashion (27, 37). One measure of gene divergence is the number of changes per unit time or the rate of change for that gene. To calculate this rate, two types of mutations, synonymous and nonsynonymous substitutions, are considered within protein coding regions. A nonsynonymous substitution is one that changes a codon and subsequently the encoded amino acid. Synonymous substitutions, or silent substitutions, change a particular codon but do not change the encoded amino acid; this is the result of degeneracy of the genetic code. While it has been proposed that mutations in bacteria occur at a regular, clock-

like rate, mutations do not accumulate at the same rate for the two types of sites (22). Nonsynonymous substitutions may have detrimental effects and thus be eliminated from the observed population. However, since synonymous substitutions do not alter the amino acid sequence, these substitutions are presumed to be selectively neutral, and they accumulate in the population over time at relatively constant rates. The percentage of synonymous substitutions is determined by dividing the number of potential synonymous sites by the number of changes at those sites. Calculation of percent synonymous base substitutions was based on the method of Li et al. (22). The rate is determined by dividing percent substitution by the evolutionary distance (time) between the two species, on the basis of 16S rRNA comparison.

Nucleotide sequence accession number. The sequence for retron ML162 was deposited into the GenBank database under accession number L36722.

RESULTS

Retron elements in the myxobacteria. On the basis of 16S rRNA sequence comparison, the myxobacteria, that is, the taxonomic order *Myxococcales*, fall into three major subgroups: *Myxococcus*, *Chondromyces*, and *Nannocystis* (34). In this study, 28 strains of myxobacteria, with representatives from each of the three major subgroups, were screened for the presence of retron elements by looking for the production of msDNA. The RT extension method was used to detect msDNA. Briefly, this technique uses Moloney murine leukemia virus RT and [α-^{32}P]dCTP to specifically radiolabel msDNA by extending the DNA strand, using the RNA strand as a template (19, 21). Extension continues to the branched guanine residue where reverse transcription stops, leaving an RNA arm at the 5′ end. Subsequent treatment of the extended and labeled product with RNase A removes the 5′ RNA arm, producing a faster-migrating species compared with the untreated sample. Figure 1 presents typical results from an RT extention assay. Strains So ce5, So ce7, So ce11, and Cm a16 (Table 1) all produced distinctly labeled bands in the size range of 145 to 245 nucleotides (Fig. 1, lanes 1 to 8). The presence of msDNA indicates the presence of the corresponding chromosomally encoded gene, the retron. The shift in mobility when the extended products were treated with RNase A (Fig. 1, lanes 2, 4, 6, and 8) is characteristic of msDNA, and the size difference reflects the length of the 5′ RNA arm. The double bands present in some strains represent either the presence of more than one msDNA or heterogeneity of extension by the RT. No labeled bands were present for strain Cb fe18 (Fig. 1, lanes 9 and 10), indicating that retron elements are absent from this *Cystobacter* strain. We screened several species of myxobacteria which have not been previously studied for the presence of retron elements, including 3 *Chondromyces*, 4 *Cystobacter*, 3 *Sorangium*, 1 *Melittangium lichenicola*, 1 *Myxococcus virescens*, 1 *Myxococcus fulvus*, 1 *Myxococcus macrosporus*, 1 *Myxococcus stipitatus*, 1 *Angiococcus disciformis*, 1 *Archangium gephyra*, 1 *Corallococcus* (*Myxococcus*) *coralloides*, and 10 *N. exedens* strains. The positive and negative strains as determined by the RT extension method are summarized in Table 1. All of the strains tested positive for the production of msDNA except for *Cystobacter ferrugineus* Cb fe18. It appears that strains Cm p5, Cm a15, and Cm a16, contain two different msDNAs, while So ce5, So ce7, and So ce11 contain only a single msDNA (data not shown). In addition, we also screened 10 *N. exedens* strains by using the RT extension assay. All 10 strains tested positive (data not shown), with the msDNAs ranging in size from 120 to 180 bases. On the basis of the different sizes of msDNAs in the *Nannocystis* strains, it is apparent that some strains may also produce more than one species of msDNA. With the exception of one *Cystobacter* strain, retrons are ubiquitous among the myxobacteria reported here (Table 1).

Homology to known retrons. Identification of retron elements similar to the two elements Mx65 and Mx162, previously identified in *M. xanthus*, was accomplished by DNA hybridization. Radiolabeled fragments derived from plasmid clones of these retrons (4–6) were hybridized to *Pst*I-digested chromosomal DNA from each myxobacterial strain. In addition, msDNA isolated from *N. exedens* Na e434 was radiolabeled and also used as a hybridization probe.

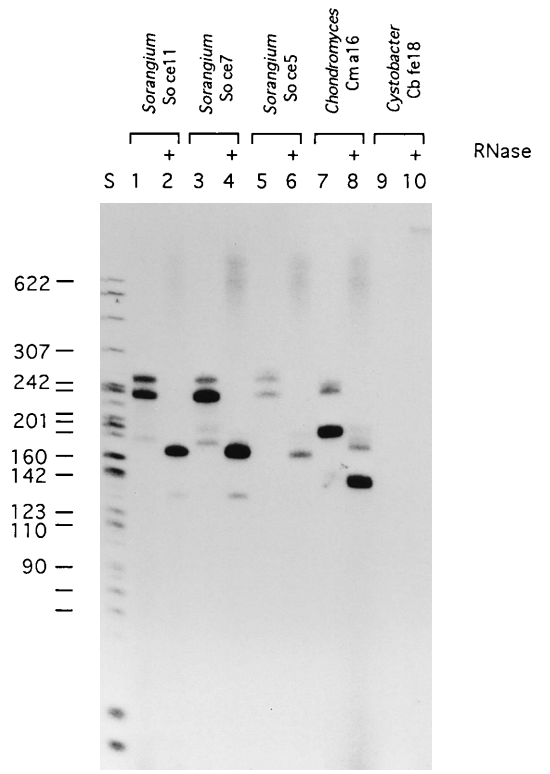


FIG. 1. Detection of ^{32}P-labeled msDNA by the RT extension assay. The molecular weight standard (S) is radiolabeled pBR322 cut with *Msp*I, and sizes are listed in base pairs. Strains are listed by genus and strain designation. Half of each labeling reaction was treated with RNase A, designated by a (+), prior to electrophoresis. Treated and untreated samples were electrophoresed in adjacent lanes. Note that RNase A-treated msDNAs (radiolabeled bands) migrate faster (lanes 2, 4, 6, and 8) than the untreated samples (lanes 1, 3, 5, and 7). Strains So ce11, So ce7, So ce5, and Cm a16 are positive for the presence of msDNA, while Cb fe18 produced no labeled band and thus is considered to be negative for msDNA production.

All of the strains in the *Myxococcus* subgroup cross-hybridized with the Mx162 probe, indicating that these species share retrons that are similar in primary nucleotide sequence to Mx162 (Fig. 2, lanes 1 to 8). In contrast, the Mx65-based probe cross-hybridized with only three species of the genus *Myxococcus* (data not shown) (Table 1): *M. xanthus*, *M. virescens*, and *M. macrosporus*. Additionally, chromosomal DNA from strains in the subgroups *Chondromyces* and *Nannocystis* did not hybridize with either the Mx162 or Mx65 probe, indicating that these retrons are restricted to the *Myxococcus* subgroup (Table 1). Figure 3 presents a phylogenetic tree of the myxobacteria showing the clustered distribution of retron elements similar to Mx162 among the seven genera that make up the *Myxococcus* subgroup. Purified msDNA, from *N. exedens* Na e434, was also used as a hybridization probe. While the *Myxococcus*, *Chondromyces*, and *Sorangium* strains did not hybridize to this probe, all 10 *Nannocystis* strains surveyed hybridized to the Na e434 msDNA probe (Table 1). This finding suggests that members of the *Nannocystis* subgroup likely contain the same, or a very similar, retron.

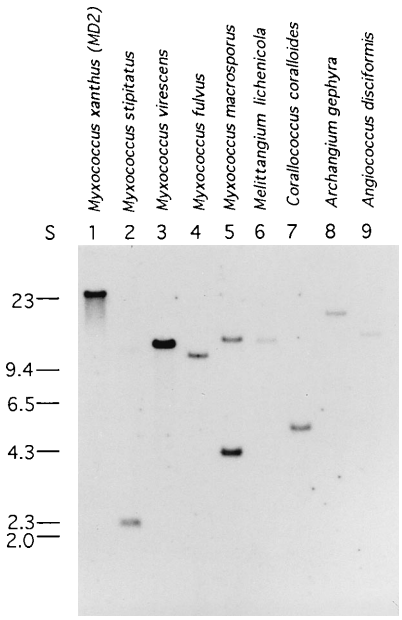


FIG. 2. Southern hybridization of nine members of the *Myxococcus* subgroup with a labeled Mx162 probe. Chromosomes were digested with *Pst*I, except for *C. coralloides*, which was digested with *Bam*HI. Digested chromosomes were electrophoresed on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled internal fragment of the RT gene from Mx162. Molecular weight standards are given in kilobase pairs. All strains tested reveal a single band which hybridized with the Mx162 probe, except *M. macrosporus*, which shows two distinct bands. Weak but clearly visible hybridization signals appear for *M. lichenicola*, *A. gephyra*, and *A. disciformis* (lanes 6, 8, and 9).

An msDNA hybridization probe was also prepared from a member of the *Chondromyces* subgroup (*Sporangium* strain So ce11). However, because of difficulty in preparing this probe, only a small number of myxobacterial strains were screened with this probe (not shown). Nevertheless, chromosomal DNA from strains So ce7, So ce11, and Cm p5 (*Chondromyces*) hybridized strongly to the msDNA probe from So ce11. DNA from *Nannocystis* strain Na e1 and *M. xanthus* DZF1 gave a weak hybridization signal, and DNA from *Nannocystis* strain Na e434 and *Melittangium lichenicola* gave no hybridization signal (not shown).

Cloning and sequencing of a *Melittangium* retron. Because the chromosome digest from *Melittangium lichenicola* gave a particularly weak hybridization signal with the Mx162 probe (Fig. 2, lane 6), we were interested in cloning and sequencing the retron from *Melittangium lichenicola* to compare sequence diversity with similar retrons found in two other genera within the same myxobacterial subgroup. The objective was to be able to make some determination about the evolutionary history of the retron, i.e., to determine whether it is an ancient element or was recently introduced into these three species.

Maxam-and-Gilbert chemical sequencing of the DNA portion of the msDNA from *Melittangium lichenicola* (data not shown) revealed the DNA strand to have 162 bases, and it has been designated ML162. ML162 msDNA showed significant homology to Mx162 in the DNA region, with only 17 base differences, 10 of which are found in the central stem region which forms a stable hairpin structure due to complementary secondary folding of the DNA strand. The majority of these base differences are complementary mutations on opposite sides of the stem that help to maintain the stem-loop structure. In addition to the nucleotide differences in the DNA, 15 of the

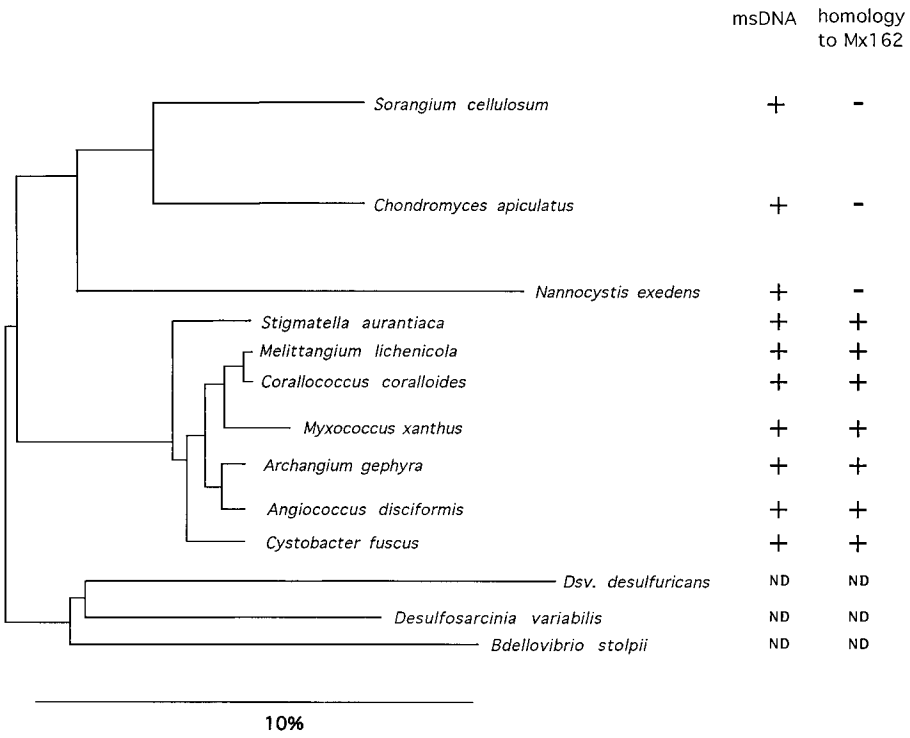


FIG. 3. Phylogenetic distribution of msDNA and Mx162 retrons in the myxobacteria. The phylogenetic tree of the myxobacteria, as determined by 16S rRNA sequencing, is abbreviated from that of Shimkets and Woese (34). Strains are listed as positive (+) or negative (−) for msDNA production and for Southern hybridization with a ^{32}P-labeled internal fragment of the RT gene from Mx162. ND indicates that the strain was not tested. It should be noted that *Desulfovibrio*, *Desulfosarcinia*, and *Bdellovibrio* strains are not myxobacteria and serve as out groups.

76 predicted nucleotides that make up the RNA strand are different.

On the basis of hybridization of Mx162 DNA to *Melittangium lichenicola* (Fig. 2, lane 6), *Pst*I chromosomal fragments of *Melittangium lichenicola* in the range of 9 to 11 kb were cloned into the *Pst*I site of pBR322. The recombinant plasmids were transformed into *E. coli* and screened by colony hybridization. One positive clone (pNaeF4.15) was picked, and confirmation of the retron clone by Southern hybridization revealed a 10.8-kb *Pst*I fragment that hybridized with the Mx162 probe. This primary clone was subsequently digested with *Bam*HI, and a 3.8-kb fragment was subcloned into the *Bam*HI site of pUC9 (pNaeBB4.0.A4). This clone was then further subcloned for sequencing.

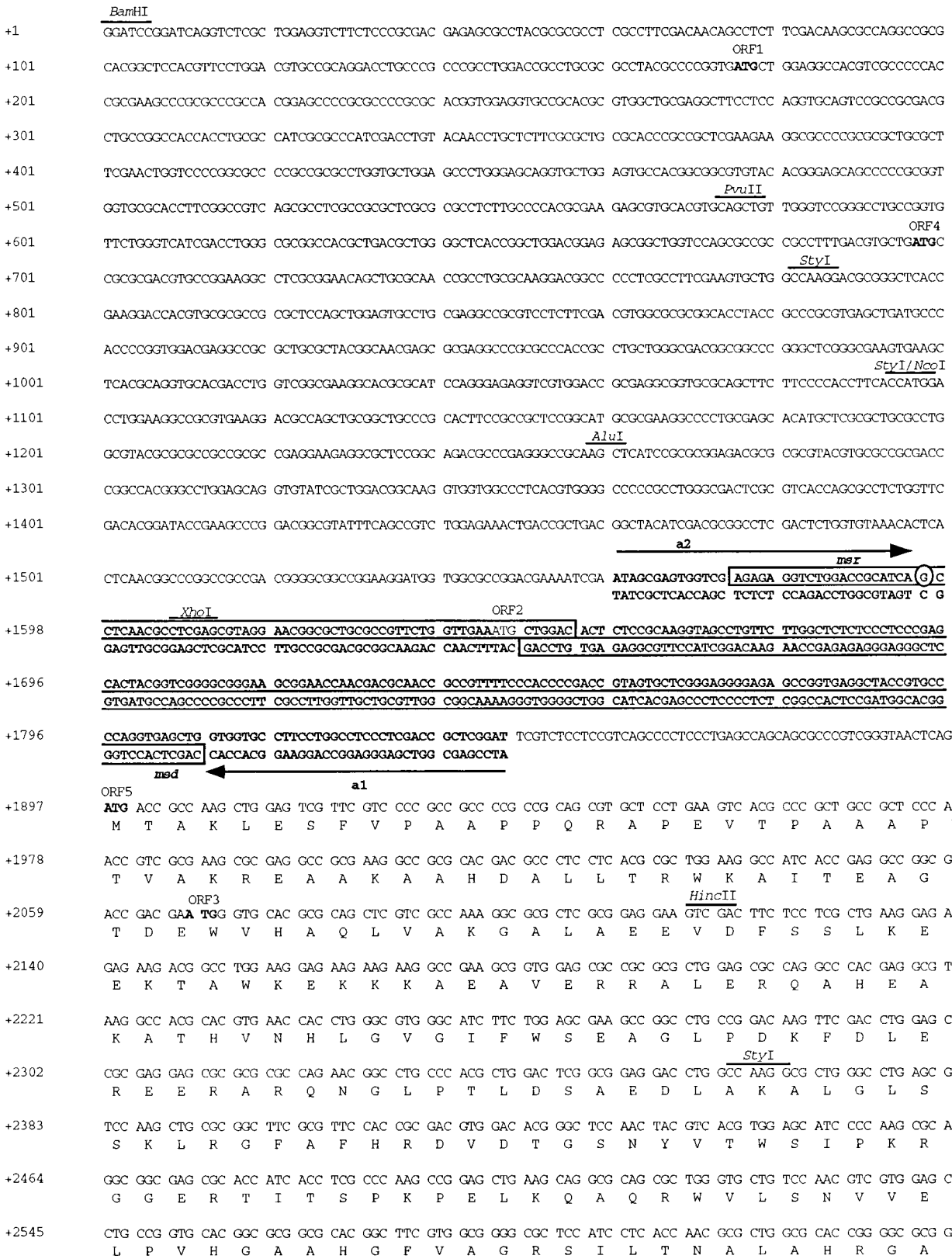
Using the Maxam-and-Gilbert method-generated sequence of the msDNA (data not shown), we were able to locate the *msd* gene from the chromosomal clone and subsequently identify the predicted *msr* gene encoding the RNA strand of the msDNA. Two 17-bp inverted repeats were found; one (a2) is located just upstream of the predicted branched guanine residue which forms the 2′,5′ RNA-DNA linkage, and the other (a1) is just downstream of the *msd* gene (Fig. 4). These inverted repeats have been demonstrated to be required for the proper folding of the primary transcript to initiate the self-priming reaction that begins msDNA synthesis (12, 18, 32). Analysis of the sequence in the vicinity of the *msr* and *msd* genes revealed five ORFs. The fifth ORF, beginning at position +1897, showed significant nucleotide identity (>70%) to the RTs found in the myxobacteria retrons Mx162 and Sa163 (13, 16). The predicted protein from ORF5 shows the seven conserved amino acid sequence domains, diagnostic for RT, including the highly conserved YADD sequence of domain 5 (40). Indeed, this ORF shows 79% nucleotide identity to the RT gene in retron Mx162. The RT ORF did not show significant nucleotide identity to the RT from Mx65. This is expected since Mx65 shows only 35% nucleotide identity to Mx162 (5, 15). The other four ORFs, ranging in size from 152 to 441 amino acids, showed no significant homology to any previously reported genes when searched through the GenBank database. Comparison of the DNA sequence from a2 to the 3′ end of the RT ORF with the Mx162 sequence revealed 77% nucleotide identity overall between the two retrons, with identity being highest in the *msr* and *msd* regions (83 to 90%) and lowest in the spacer region which is located between *msd* and the RT ORF (39.7%).

The G+C content of the myxobacteria is 67 to 72% (28). This high G+C content is reflected in the preferential usage of codons that are GC rich. The RT ORF of the retron found in *Melittangium lichenicola*, like most known genes from *M. xanthus*, shows a heavy bias toward codons that use G and C over A and T. For example, there are six possible codons for the amino acid leucine (CTA, CTC, CTG, CTT, TTA, and TTG), yet of the 48 leucine residues in the RT ORF of ML162, 98% use either CTC or CTG. There is a similarly strong preference observed among 20 known genes of *M. xanthus*, in which 88% of all leucine codons are either CTC or CTG (33). Highly statistically significant, nonrandom codon usage within the RT gene is likewise reflected in the large chi-square value for leucine codons of ML162 (not shown). This bias toward GC-rich codons is pervasive throughout the RT ORF, and it is similar to the codon preference observed among other known genes of *M. xanthus* and consistent with an organism with a high-G+C genome. Such a GC codon bias has also been noted previously for the RT ORF of retron Mx162 (16).

Retron junctions. At present, the precise ends of retrons in the myxobacteria are unknown. Therefore, the ends of a retron element can be defined only as unique DNA sequences which are associated with the genes encoding msDNA and RT. If two similar or isogenic chromosomes exist, in which one of the chromosomes lacks the retron element, then comparison of the corresponding region between the two chromosomes should reveal the junctions of the retron element. However, for *M. xanthus*, there are no known strains in which the Mx162 retron element is absent. Alternatively, a different strategy can be used to identify the ends of this genetic element by comparison with the ML162 retron from *Melittangium lichenicola*. Since the two retrons from *Melittangium lichenicola* and *M. xanthus* are very similar in DNA sequence, and if the chromosomal regions in which these elements have inserted are significantly different in the *Melittangium* and *Myxococcus* DNA sequences, then it could be possible to approximate where the 5′ and 3′ ends of the element lie. Figure 5 presents an alignment of flanking DNA sequence from *M. xanthus* and *M. lichenicola* around the 3′ end of the RT ORFs of their respective retrons. In this 3′ region, sequence identity rapidly degenerates from >70% to almost zero and extends for several hundred base pairs of sequence downstream from the RT ORF. This 3′ junction appears to be very near the stop codon of the RT ORF (Fig. 5). Interestingly, at the 5′ end of the retron, significant nucleotide identity is maintained over 300 bp upstream of the *msr* gene. This indicates that the retron could be inserted in a gene common to *M. xanthus* and *Melittangium lichenicola* or probably in some homologous location on each chromosome. Alternatively, the retron could have been transmitted vertically from a common ancestor of *M. xanthus* and *Melittangium lichenicola*, in which case the junction sequences would not be distinguishable from host sequences if the two species diverged at similar rates. A similar situation is observed with the DNA sequence flanking the retron Sa163 from *S. aurantiaca* (13), in which case significant nucleotide identity is maintained upstream of the *msr* gene but not downstream of the RT gene.

Sequence divergence. By calculating the rate of nucleotide change at synonymous sites for the RT gene encoded by the Mx162-type retrons, the divergence of this gene can be compared with that of other bacterial genes. Such comparisons may provide a hint about the origin and age of this RT gene found in the myxobacterial genome.

The average rate of silent substitution found for genes common to *Salmonella* species and *E. coli* is 0.335 substitutions per million years (31) (Table 2). The fastest and slowest rates of substitution between *E. coli* and *Salmonella* species are 0.63 substitutions per million years and 0.08 substitutions per million years, respectively. The *Melittangium* and *Myxococcus* myxobacteria are estimated to have diverged from a common ancestor about 90 million years ago. This estimate is based on comparison of their 16S rRNA sequences (34) and a universal substitution rate calculated for bacterial genomes (27). The percent divergences of the RT genes from retrons Mx162 and ML162 were compared by the method of Li et al. (22) (Table 2). ML162 and Mx162 show 46% divergence at silent sites, which translates into a rate of 0.26 silent substitutions per million years. This is close to the average rate of change (0.335) for genes reported in *Salmonella* species and *E. coli*, which presumably diverged about 140 million years ago (31). In fact, it can be argued that the degree of sequence variation observed among the Mx162 RT genes is more significant than what is reflected in the calculated rate of silent substitutions. This is because of the high G+C content of the myxobacterial genome, which results in strong codon bias in protein-coding regions. This in turn may reduce the rate at which substitutions at silent sites accumulate, by limiting the number of alternative codons that can be used. Thus, the amount of sequence vari-


BamHI
ORF1
PvuII
ORF4
StyI
StyI/NcoI
AluI
a2
msr
XhoI
ORF2
msd
a1
ORF5
ORF3
HincII
StyI

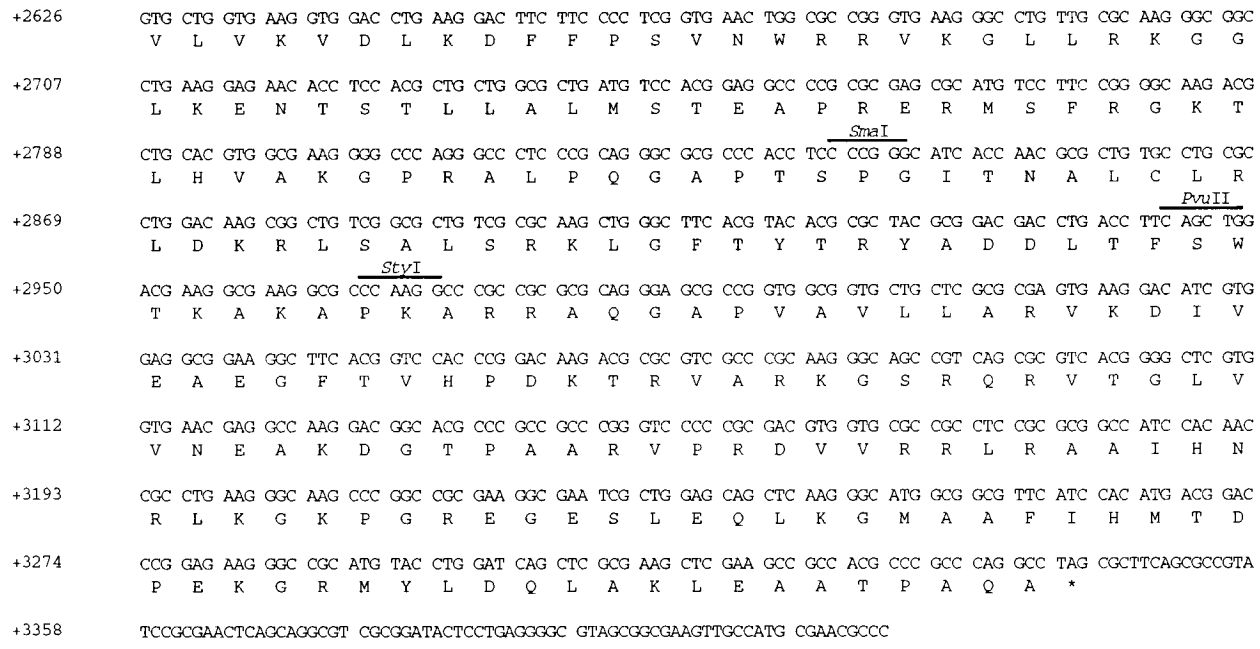


FIG. 4. Complete nucleotide sequence of retron ML162 from *Melittangium lichenicola* (GenBank accession number L36722). Potential ORFs are labeled, and the ATG start codons are in boldface. The msDNA coding region for ML162 starts at nucleotide +1662 and includes the RNA (*msr*) and DNA (*msd*) coding regions for msDNA (boxed), the branched guanine residue at position +1597 (circled), and the a2 and a1 inverted repeats at +1662 and +1841 (arrows). The RT ORF (ORF5) begins at +1896 and ends at position +3342.

ation observed between the RT genes of *Myxococcus* and *Melittangium* species, although relatively small, is probably evolutionarily significant, having accumulated changes at the same rate as most other genes since the divergence of the genera *Myxococcus* and *Melittangium* from a common ancestor.

DISCUSSION

It is evident from findings presented here and previously (4, 19, 29) that the family of retrons found in the myxobacteria are unique compared with retrons found in other bacteria and that the origin and acquisition of these retrons in the myxobacteria may be different from those of the other proteobacteria. The first indication is based on the distribution of retrons in the myxobacteria compared with other proteobacteria, such as *E. coli*. Retrons are ubiquitous, or nearly so, in the myxobacteria, being absent in only one *Cystobacter* strain out of 28 myxobacterial strains tested in this study. In contrast, only about 10% of all *E. coli*, *Klebsiella*, *Salmonella*, *Proteus*, *Rhizobium*, and *Bradyrhizobium* strains tested have been demonstrated to contain retrons (11, 29). The distribution of a particular retron type within the myxobacteria appears to cluster within a phylogenetic subgroup (Fig. 3). For example, elements very similar to retron Mx162 (originally discovered in *M. xanthus*) were found in five different species of *Myxococcus* and three species of *Cystobacter* as well as in *Melittangium lichenicola*, *C. coralloides*, *Archangium gephyra*, *Angiococcus disciformis*, and previously *S. aurantiaca* (Table 1) (9). On the basis of 16S rRNA analysis (34), all of these bacteria form a phylogenetically related cluster or subgroup within the myxobacteria (Fig. 3). Likewise, all 10 strains of the *Nannocystis* subgroup surveyed appear to share the same novel retron element (Table 1).

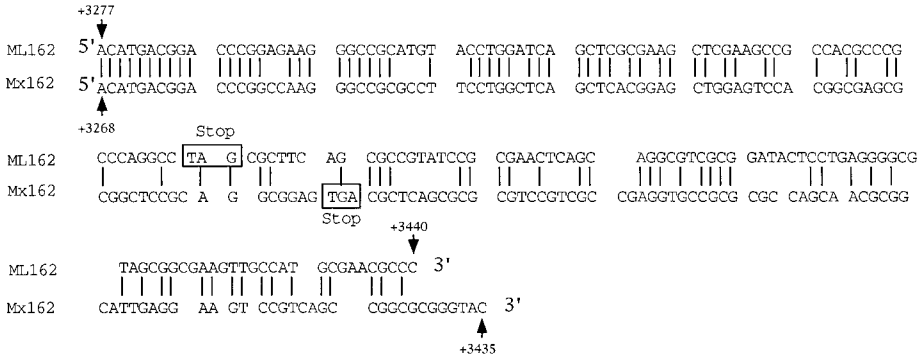


FIG. 5. DNA sequence alignment of the 3′ ends of ML162 and Mx162. The alignment was created by using the Bestfit program of the Genetics Computer Group software (2). Stop codons for each RT-coding sequence are boxed. The sequences shown begin at positions +3277 and +3268 for ML162 and Mx162, respectively, and end at positions +3440 and +3435. A close match between the two sequences appears to end around the stop codon for their respective RT ORFs.

TABLE 2. Rates of change at silent sites for RT and other bacterial genes

Organisms compared	Time of divergence (million yr ago)	Gene	% Divergence at silent sites (corrected)	Rate of substitution at silent sites/million yr
Melittangium sp.-*Myxococcus* sp.	90	RT	46	0.26
E. coli-Salmonella sp.	140	*trpA*	177	0.63
	140	*ptsH*	23	0.08
	140		94 (avg)	0.335 (avg)

This clustered distribution of the Mx162 retron within the *Myxococcus* subgroup implies a vertical rather than a horizontal transmission of this particular element. In other words, an evolutionary scenario can be envisioned in which the genome of a common ancestor acquired an Mx162-type retron element, with subsequent inheritance of this element by all the different genera during the divergence and evolution of this subgroup.

Other evidence also tends to support this scenario. For example, a homolog of retron Mx162 found in *Melittangium lichenicola*, retron ML162, was cloned, and its DNA sequence was compared with sequences of two previously characterized examples of this retron type (Mx162 and Sa163). Overall nucleotide identity between retrons Mx162 and ML162 is 77%. In addition, the silent site substitution rate for the RT ORFs from these two retrons is calculated to be 0.26 substitutions per million years (Table 2). These figures are consistent with the view that these two genes have diverged for at least as long as the genera *Myxococcus* and *Melittangium* shared a common ancestor, for about 90 million years ago.

In contrast, when the retron elements found in other proteobacteria such as *E. coli* are compared with the findings presented here for the myxobacteria, it appears unlikely that the Mx162 retron is a recent acquisition by the myxobacterial genome. For example, in *E. coli*, the retron element Ec107 is widely distributed among ECOR strains, a reference collection of natural isolates (10, 11, 17). However, its occurrence is sporadic, and this element is found in distantly related phylogenetic branches of the ECOR collection. Also, DNA sequence determination of several individual examples of the Ec107 element revealed little or, in some cases, no nucleotide sequence diversity among these retrons (17). In addition, codon usage within the RT ORFs of most *E. coli* retrons is atypical for native *E. coli* genes (16). All of these features appear to mark the Ec107 retron as a recent addition to the genome of certain lineages of *E. coli*, with the likely horizontal spread to other strains.

The features described here for the Mx162 class of retron found in the myxobacteria do not resemble those of the *E. coli* retrons and appear to be inconsistent with a recent inheritance in the genome. Indeed, an analysis of the codon usage for the RT ORF from the retron of *Melittangium lichenicola* revealed a heavy bias toward codons that use predominantly G and C, and this usage is typical of other known myxobacterial genes (16, 33). Although recent acquisition from a GC-rich organism cannot be discounted, this characteristic does suggests that the retron-encoded RT gene has resided in the myxobacterial genome for millions of years, at least since the speciation of the *Myxococcus* subgroup. However, such inheritance may not be true for other myxobacterial retrons. Retron Mx65 was found in only two species other than *M. xanthus* and may have been introduced into or lost from the *Myxococcus* genome at a later time.

Evidence continues to indicate that, as in the eukaryotes, RT-encoding genetic elements of diverse types are probably widespread and a nascent characteristic of the prokaryotic world. This idea is supported by the prevalence of diverse retron elements, among a wide variety of bacteria, as documented here and in a previous report (29). Additional evidence comes from the recent discovery of RT-encoding group II introns in several groups of the eubacteria (8). Many questions about the retron elements need to be answered, including their origin in the bacterial genome, potential mobility, and relationship with other classes of retroelements. On the basis of the prevalence of RT genes, has the process of reverse transcription played a role in the generation of genetic variation or otherwise influenced the evolution of the bacterial genome? Indeed, the chromosomes of the myxobacteria appear to have a particular predilection for retron elements and may be a good place to look for clues to answer these questions. Because retrons can be deleted from the chromosome of *M. xanthus* without affecting their growth or development in the laboratory, no function has been linked to these genetic elements. It cannot be discounted, however, that these RT-encoding elements have a function which has provided a selective advantage for the myxobacteria. This could be an alternative explanation for why retron elements have been retained by the myxobacterial genome for millions of years.

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