Bacterial clade with the ribosomal RNA operon on a small plasmid rather than the chromosome

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rRNA is essential for life because of its functional importance in protein synthesis. The rRNA (*rrn*) operon encoding 16S, 23S, and 5S rRNAs is located on the "main" chromosome in all bacteria documented to date and is frequently used as a marker of chromosomes. Here, our genome analysis of a plant-associated alphaproteobacterium, *Aureimonas* sp. AU20, indicates that this strain has its sole *rrn* operon on a small (9.4 kb), high-copy-number replicon. We designated this unusual replicon carrying the *rrn* operon on the background of an *rrn*-lacking chromosome (RLC) as the *rrn*-plasmid. Four of 12 strains close to AU20 also had this RLC/*rrn*-plasmid organization. Phylogenetic analysis showed that those strains having the RLC/*rrn*-plasmid organization represented one clade within the genus *Aureimonas*. Our finding introduces a previously unaddressed viewpoint into studies of genetics, genomics, and evolution in microbiology and biology in general.

ribosomal RNA operon | chromosome | plasmid | genome rearrangement | *Aureimonas*

Multipartite genomes containing more than one chromosome are not unusual in bacteria. The presence of the main (i.e., largest) chromosome and the second chromosome(s) in one genome is conserved among all members of some genera, such as *Burkholderia* and *Vibrio*. Chromosomes are distinguished from plasmids by the localization of essential genes (1, 2). In general, main and second chromosomes are larger (>0.5 Mb) than coresident plasmids, have similar guanine+cytosine (G+C) contents to each other, and are maintained by cell cycle-linked replication and active partitioning systems, whereas plasmids are diverse in that some (*i*) can be conjugally mobilized, (*ii*) can replicate within one genus or within many genera (i.e., have a broad host range), (*iii*) are present in high copy numbers and are segregated stochastically, and (*iv*) have lower G+C contents than their host chromosomes (1–3). One of the practical ways of designating a replicon as a "chromosome" has been to examine the localization of the rRNA (*rrn*) operon, which encodes rRNAs (16S, 23S, and 5S rRNAs), because of its functional importance in protein synthesis. Additional copies of the *rrn* operon are also sometimes found on the second chromosomes (e.g., in species of *Rhodobacter*, *Brucella*, *Burkholderia*, and *Vibrio*) (4–7) or on plasmids (the 53.9-kb and 23-kb plasmids of *Bacillus megaterium* and *Paracoccus* species, respectively) (8, 9). However, so far, no documented bacterial strain lacks the *rrn* operon on the "main" chromosome.

Here, we report genome analysis of the genus *Aureimonas*, showing that one clade within this genus carries its sole *rrn* operon on a small plasmid.

Results and Discussion

Plasmid Localization of the *rrn* Operon in *Aureimonas* sp. AU20. The genus *Aureimonas* (family Aurantimonadaceae, order Rhizobiales, class Alphaproteobacteria) contains three defined species, *Aureimonas altamirensis*, *Aureimonas ureilytica*, and *Aureimonas frigidaquae*, and its members have been isolated from diverse environments (10). To characterize this bacterial group from a genomic viewpoint, we determined the complete genome sequence of *Aureimonas* sp. AU20, an isolate from the stem of a soybean plant (11). The result showed that the AU20 genome (5.2 Mb in total) contains nine circular replicons: the main chromosome (3.7 Mb) and eight other replicons (designated pAU20a to pAU20g and pAU20*rrn*) (Table 1 and Fig. S1). The five smallest replicons, pAU20d to pAU20g and pAU20*rrn*, could be distinguished from the chromosome by their lower G+C contents, suggesting that they had distinct evolutionary origins. The genome contained a single *rrn* operon, 55 tRNA genes, and 4,785 protein-coding genes (Table 1). Surprisingly, the *rrn* operon, which consisted of genes for 16S rRNA, $tRNA^{Ile}$, $tRNA^{Ala}$, 23S rRNA, and 5S rRNA, was located not on the chromosome but on the smallest replicon (9.4 kb), pAU20*rrn*. The $tRNA^{Ile}$ gene on this replicon was also the only one in the genome. The $tRNA^{Ala}$ gene on pAU20*rrn* showed similarities to the $tRNA^{Ala}$ gene and the $tRNA^{Thr}$ gene on the chromosome (82% and 78% identical, respectively; Fig. S2*A*), but the two replicons shared no other substantial similarities. Using the 16S rRNA gene as a probe, we performed Southern hybridization analysis of the genomic DNA that was digested with the enzyme I-*Ceu*I, an endonuclease whose cleavage site exists exclusively within the 23S rRNA gene (12) (Fig. 1*A*). The result showed a single 9-kb band, supporting the localization of the *rrn* operon on pAU20*rrn*. A single hybridization band was also detected with EcoRI-digested DNA, consistent with the existence of a single *rrn* operon in the genome. This *rrn* operon (with a size of 6 kb) had the typical core promoter consisting of the putative UP element and −35 and −10

Significance

In bacterial genomes, chromosomes are distinguished from plasmids by the localization of essential genes. It has been taken for granted that fundamental genes such as the rRNA (*rrn*) operon should be transmitted faithfully on the chromosome. Here, we found a striking exception: A plant-associated bacterium, *Aureimonas* sp. AU20, and its close relatives harbor the *rrn* operon only on a small, high-copy-number replicon but not on the chromosome. Our findings show the existence of novel genome organization in bacteria.

Author contributions: M.A., Y.O., T.O., Y.N., K.M., and H.M. designed research; M.A., Y.O., and T.O. performed research; Y.O. led the AU20 genome finishing and developed computational tools; M.A., Y.O., T.O., and H.M. analyzed data; and M.A., Y.O., T.O., M.S., Y.N., M.T., K.M., and H.M. wrote the paper.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database, www.ncbi.nlm.nih.gov/genbank/, and in the DNA Data Bank of Japan database, www.ddbj.nig.ac.jp. A list of accession numbers is provided in Table 1 and Table S2.

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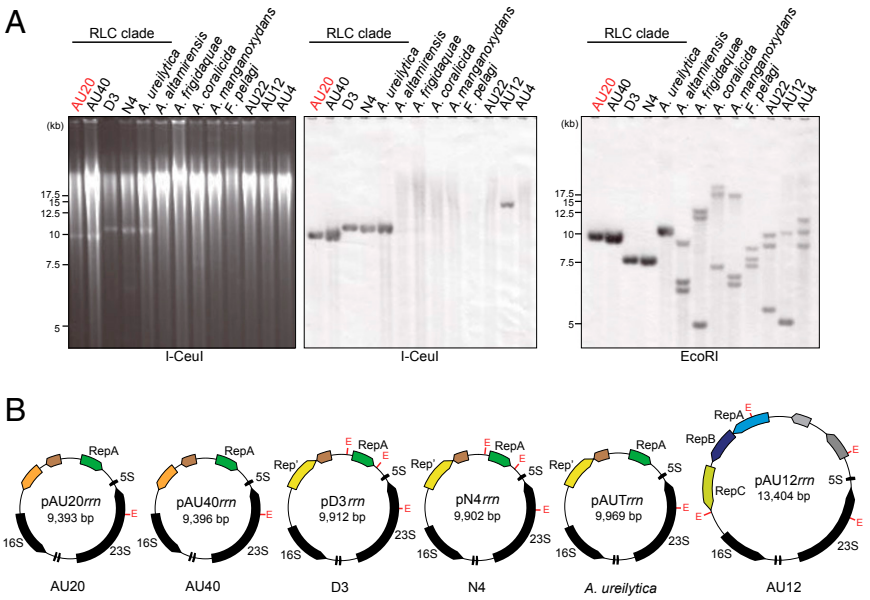

Fig. 1. The *rrn* operons in members of the family Aurantimonadaceae. (*A*) Southern hybridization analysis. Total DNA was digested with I-*Ceu*I, separated by electrophoresis (*Left*), transferred to a membrane, and probed with the 16S rRNA gene (*Middle*). (*Right*) EcoRI-digested total DNA was separated by electrophoresis, transferred, and probed with the same gene. One of the bands predictable from the genome sequence was not detected in the EcoRI-digested DNA of AU12 and AU4 (corresponding to a 34.3-kb band and a 48.2-kb band, respectively). (*B*) Maps of the *rrn*-plasmids in strains AU20, AU40, D3, N4, and *A. ureilytica* and another type of *rrn*-carrying replicon in AU12. Positions of EcoRI sites are marked with a red "E." The rRNA and tRNA genes are shown in black. RepA and Rep′ encoded on the *rrn*-plasmids and RepC on pAU12*rrn* are homologs of different replication initiators. RepA and RepB on pAU12*rrn* are homologs of partitioning proteins.

consensus hexamers (13) (Fig. S2*B*) and a putative rho-independent terminator (Fig. S2*C*).

The regions of the origin and terminus of replication of the chromosome (*oriC* and *ter*, respectively) were not unusual in alphaproteobacteria (Fig. S3). In contrast, the replication system of pAU20*rrn* was similar to the replication system of pPS10, a 10-kb high-copy-number plasmid isolated from *Pseudomonas syringae* that has a stable host range confined to *Pseudomonas* species (14–16). The pAU20*rrn*-encoded RepA protein showed similarity to the pPS10 RepA protein in both primary (37% identical in amino acid sequence) and secondary structures (Fig. 1*B* and Fig. S4*A*), suggesting that the two RepA proteins behave similarly in replication control. In the case of pPS10, the RepA monomer initiates replication, whereas the RepA dimer represses the transcription of its own gene (16, 17). Upstream of *repA* on pAU20*rrn* (a predicted *oriV* site) were three tandem repeats (iterons) that formed a putative site for the binding of RepA monomers and an inverted repeat that is a putative site for the binding of the RepA dimer. In addition, an AT-rich region and a putative DnaA box (Fig. S4*B*) could be involved in the initiation of replication. In a quantitative PCR (qPCR) analysis, we determined that the number of pAU20*rrn* copies in the cell varied from 18 to 34 per chromosome equivalent throughout the growth phases (Fig. S4 *C* and *D*). The deduced amino acid sequences of the two ORFs present outside the *rrn* and *repA*/*oriV* regions did not have any motifs that are conserved among proteins involved in active partitioning of replicons, suggesting the stochastic segregation of pAU20*rrn*.

Taken together, pAU20*rrn* does not match the criteria of a second chromosome because of its small size, high copy number, and lack of partitioning genes. It also differs from *rrn*-carrying plasmids of *B. megaterium* and *Paracoccus* species in that it alone carries the genes for rRNAs and $tRNA^{Ile}$ within the genome. Therefore, pAU20*rrn* represents a distinct class of replicons, which we designated the *rrn*-plasmid, which is coupled with an *rrn*-lacking chromosome (RLC).

Distribution of the *rrn*-Plasmids in the Family Aurantimonadaceae. To examine whether this RLC/*rrn*-plasmid genomic organization was specific to AU20, we determined the draft genome sequences of an additional 12 strains in the family Aurantimonadaceae (Tables S1 and S2). We identified *rrn* operons with the same organization as the organization of AU20 (including genes for $tRNA^{Ile}$ and $tRNA^{Ala}$)

Table 1. General features of the *Aureimonas* sp. AU20 genome

Replicon	Size, bp	G+C content, %	tRNA gene	rRNA operon	Protein-coding gene	Accession no.
Chromosome	3,742,793	66.8	53	0	3,449	CP006367
pAU20a	488,888	67.8	0	0	425	CP006368
pAU20b	436,017	67.0	0	0	405	CP006369
pAU20c	311,483	68.1	0	0	277	CP006370
pAU20d	101,355	61.0	0	0	98	CP006371
pAU20e	57,210	59.3	0	0	54	CP006372
pAU20f	35,461	61.8	0	0	32	CP006373
pAU20g	28,258	61.2	0	0	42	CP006374
pAU20*rrn*	9,393	58.0 (61.2*)	2	1	3	CP006375
Total	5,210,858	—	55	1	4,785	—

*Value obtained from a 3.3-kb segment of the replicon when stripped of the *rrn* operon.

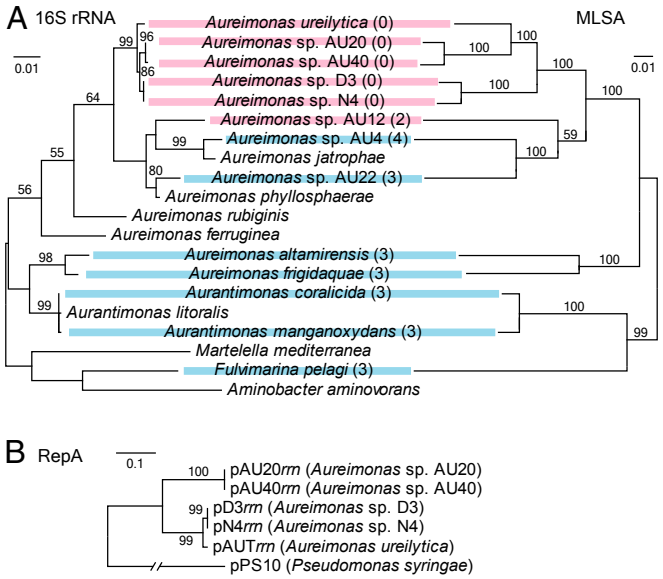

Fig. 2. Phylogenetic relationship between members of the family Aurantimonadaceae using the maximum likelihood method. (*A*) Phylogenies based on nucleotide sequences of the 16S rRNA gene (*Left*) and the chromosomal genes *atpD, dnaK, gyrB, rpoB,* and *rpoC* concatenated by multilocus sequence analysis (MLSA) (*Right*). Colored lines on the back of strain names indicate that the strains were used in both trees; strains with red lines harbor the *rrn* operon on extrachromosomal replicons. A numeral in parentheses after each strain name is the number of the chromosomal *rrn* operons. (*B*) Relationship of the RepA amino acid sequence among the *rrn*-plasmids and pPS10, with their host names in parentheses. Numerals above branches are the related bootstrap values (%; values ≥ 50 are shown). Scale bars indicate the substitution number per site.

in all of the strains (the region upstream of the 16S rRNA gene and a putative terminator are shown in Fig. S2). Four of the strains (*A. ureilytica* and *Aureimonas* spp. AU40, D3, and N4) (11, 18, 19) were similar to AU20 in that their *rrn* sequences were assembled into small (9.4–10 kb) circular replicons that contained pPS10 *repA*-like genes and *oriV* regions similar to pAU20*rrn* (Fig. 1*B* and Fig. S4 *A* and *B*). Southern hybridization analysis of the I-*Ceu*I– or EcoRI-digested DNA of each of the four strains resulted in the detection of a single band when using the 16S rRNA gene as a probe (Fig. 1*A*). Therefore, we considered their *rrn*-carrying replicons to be *rrn*-plasmids.

In each of the other eight strains, three or four different contigs were initially linked to the same *rrn* sequence on both its upstream and downstream sides. We investigated which pair of upstream and downstream contigs actually sandwiched the *rrn* operon by using PCR with primers designed from these contigs. This investigation clarified all of the *rrn*-flanking contigs in each genome (Tables S3 and S4; the number of *rrn* operons determined in this way is shown in Fig. 2). The assembled *rrn*-containing contigs/scaffolds, except for one contig in *Aureimonas* sp. AU12, were much longer (≥89.5 kb) than the *rrn*-plasmids. Moreover, one of these contigs/scaffolds in each strain (except *Fulvimarina pelagi*) contained a predicted *oriC* site, a chromosomal hallmark. Therefore, we regarded those *rrn* operons as chromosomally located, with such operons in each strain designated as *rrnA*, *rrnB*, *rrnC*, and/or *rrnD*. Southern hybridization analysis of the EcoRI-digested DNA of the eight strains confirmed the presence of different copies of the 16S rRNA gene, because all copies of this gene sequenced in this study lacked the EcoRI site (Fig. 1*A*). More intense hybridization signals from the *rrn*-plasmids than from the chromosomal signals suggested high copy numbers of the *rrn*-plasmids per chromosome (Fig. 1*A*). One of the three *rrn*-flanking sequences in AU12 was assembled into an exceptional 13.4-kb circular replicon, pAU12*rrn* (Fig. 1*B* and Table S3). Unlike pAU20*rrn*, this replicon carried the *repABC* cassette, which encodes the replication initiator RepC and the RepA-RepB–based active partitioning system (20).

Phylogeny of the Strains Having the *rrn*-Plasmids. In both the phylogenetic analysis using 16S rRNA genes and the phylogenetic analysis using five housekeeping protein-coding genes, *atpD*, *dnaK, gyrB*, *rpoB*, and *rpoC* (all chromosomally located in AU20) (Fig. 2*A* and Table S4), the five strains with the RLC/*rrn*-plasmid organization were clustered into one clade (designated the RLC clade) within the genus *Aureimonas*. The similar topology of the two phylogenetic trees suggested that all of the *rrn* operons on the *rrn*-plasmids originated evolutionarily from the common ancestor of the clade. In contrast, the *rrn*-plasmids in the RLC clade seemed to have diverged themselves after the origination of the first *rrn*-plasmid (or, alternatively, had independent origins) because they split into two subgroups of AU20/AU40 and D3/N4/*A. ureilytica* with regard to the region outside the *rrn* operon. RepA was ≥97% and ≤75% identical within each subgroup and between subgroups, respectively (the RepA genealogy is shown in Fig. 2*B*). Moreover, the latter subgroup encoded a protein (labeled Rep′ in Fig. 1*B*) with similarity to the Rep_3-type replication initiator of a *Zymomonas mobilis* plasmid (21).

Chromosome Rearrangements Observed in the Genus *Aureimonas*. We compared the AU20 chromosome with chromosomal *rrn* contigs from strains AU4, AU12, and AU22, which are non-RLC members close to the RLC clade in the genus *Aureimonas*. This analysis showed several synteny breaks around each of *rrnA*, *rrnB*, and *rrnC* (Fig. S5). In the *rrnC* region, tRNA genes were situated at some of the breakpoints (Fig. S5 *C* and *F*), suggesting that the tRNA genes were involved in chromosome inversion and integration of alien sequences to generate chromosome variation. In the *rrnA* and *rrnB* regions, any characteristic sequences were not found at the synteny breakpoints (Fig. S5 *A*, *B*, *D*, and *E*). Apparent frequent recombination around *rrnC* might be related to the *ter* region, where a stalled replication fork is potentially subject to DNA cleavage by some endonucleases (e.g., as type I and type IV restriction enzymes) and subsequent recombinational repair (22, 23). However, we failed to find any sign of recombination that could explain the loss of the *rrn* operon; the comparisons between more closely related RLC and non-RLC strains would serve to trace their common ancestral chromosome and the process toward the RLC/*rrn*-plasmid organization.

Implication of the *rrn*-Plasmid for Bacterial Genomics. What evolutionary significance does the emergence of the RLC clade involve? It can first be said that their members have acquired increased rRNA gene dosage. In general, the number of *rrn* operons on the chromosomes of diverse bacterial species and strains varies, irrespective of their whole-genome sizes; for example, the *Bradyrhizobium diazoefficiens* genome (9.1 Mb) contains only one copy (24), whereas the genomes of *Escherichia coli* (4.6 Mb) and *Bacillus subtilis* (4.2 Mb) contain seven and 10 copies, respectively (25, 26). In *E. coli*, the presence of multiple *rrn* operons facilitates a sudden increase in the rate of rRNA synthesis, enabling rapid adaptation to nutritional upshift or favorable temperature shift (27). Therefore, we consider it possible that the high copy number of the *rrn*-plasmid, compared with four *rrn* copies at most on the chromosome, confers a selective advantage to the *Aureimonas* host under changing environmental conditions. In this context, it is noteworthy that the RLC clade overlaps completely with the operational taxonomic unit whose relative abundance in soybean-associated bacterial communities fluctuates drastically in response to phenotypic and nutritional changes of the host plants (11, 28). Second, split of the *rrn* operon from the chromosome possibly has an additional regulatory influence, for example, on the

global transcription profile by redirecting RNA polymerase from the chromosome to the separate replicon. In this sense, it will be interesting to see a subcellular localization pattern of the *rrn*-plasmid. In eukaryotes, the nucleolus displays functionality of a distinct subnuclear compartment, which is formed around the ribosomal DNA. The nucleolus functions not only in ribosome biogenesis but also in regulation by sequestering key factors of cell-cycle progression and stress responses into the compartment (29). Third, localization of the *rrn* operon on a small plasmid has possibly lowered a barrier to its horizontal transfer, facilitating its exchange between bacteria residing in common niches. Several studies have indeed suggested the horizontal transfer of the 16S rRNA genes (30). The replication host range and the potential for conjugal transfer of the *rrn*-plasmid will be very interesting subjects in future studies.

We conducted a survey of the complete genome sequence database for the RLC/*rrn*-plasmid organization in a wide range of bacterial phyla. We found that *Butyrivibrio fibrisolvens* 16/4, butyrate-producing bacterium SS3/4 (phylum Firmicutes), and *Fretibacterium fastidiosum* SGP1 (phylum Synergistetes) have main chromosomes devoid of rRNA-coding genes, although the predicted coresident *rrn*-carrying replicons have not been included in the database. This result raises the possibility that RLC is not limited to the genus *Aureimonas*. Altogether, our findings pose a fundamental question of what has shaped the evolution of multipartite genomes in life. In environmental microbiology, moreover, the existence of the RLC/*rrn*-plasmid organization should be taken into consideration when profiling microbial communities on the basis of rRNA gene sequences.

Methods

Total DNA Extraction. Total DNA was extracted with a DNeasy Blood & Tissue Kit (QIAGEN) in accordance with the manufacturer's instructions, with slight modification (11).

Determination and Annotation of the AU20 Genome Sequence. A fragmented single-end genome library was sequenced by using 454 GS-FLX Titanium (Roche), producing 236,422 reads (92 Mb in total). Then, a 3-kb mate-pair genome library was sequenced by using a HiSeq 2000 (Illumina) instrument (operated by Eurofins Genomics, Inc.). The obtained reads were trimmed based on underrepresented 21-mers by using ShortReadManager (31), and 1,000,000 of the resulting reads (79 Mb in total) were used for subsequent assembly. Finally, an 8-kb paired-end genome library, which was constructed by Takara-Bio, Inc., was sequenced by using 454 GS-FLX Titanium, producing 262,026 reads (121 Mb in total). The reads from these three systems were assembled by using Newbler, version 2.6 (Roche). The finishing was facilitated by using GenoFinisher (31) and AceFileViewer (31), and resulted in the generation of eight circular contigs and one large scaffold containing six gaps. To close all of the gaps, DNA fragments encompassing the gaps were amplified by PCR with primers designed by GenoFinisher, and the reaction products were sequenced with the Sanger method by using a 3730xl DNA Analyzer with a BigDye Terminator Cycle Sequencing Reaction Kit (Life Technologies).

The genome sequence was annotated by using the National Center for Biotechnology Information (NCBI) Prokaryotic Genomes Automatic Annotation Pipeline (32), and the result was manually inspected with respect to positions of start codons for predicted ORFs by using the Microbial Genome Annotation Pipeline (MiGAP; www.migap.org/) and GenomeMatcher (33). All of the ORFs were translated into amino acid sequences, which were then subjected to similarity searches against the RefSeq collection from the NCBI by using BLAST2GO (34) (with an Expect value $<10^{-3}$ for cutoff). Protein domains were identified by using InterProScan (35).

Southern Hybridization. Total DNA (1.5 μg) digested with I-*Ceu*I or EcoRI was electrophoresed in a 0.8% agarose gel and blotted onto a nylon membrane. The blot was probed with a fragment of the *rrs* gene (encoding 16S rRNA) that was amplified from the AU20 DNA by PCR using the primer pair 5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-AAGGAGGTG(A/T)TCCA(A/G)CC-3′ and labeled with digoxigenin (DIG). Probe labeling, hybridization, and signal detection were performed with a DIG Labeling and Detection Kit (Roche).

Determination of the pAU20*rrn* Copy Number. An AU20 culture was grown, with vigorous shaking, at 28 °C in tryptone-yeast extract medium, which contained tryptone (5 g/L), yeast extract (3 g/L), and $CaCl_2{\cdot}2H_2O$ (0.83 g/L). Aliquots were taken at intervals and centrifuged to collect the cells for DNA extraction. We selected *rpsB*, which was located close to *ter* on the chromosome, as a target chromosomal gene for qPCR, and used the primer pair 5′-TGCTGACGAACTGGAAGACG-3′ and 5′-GCAGGTTGAGACGCTCCTTC-3′ to amplify a 112-bp product. We selected *rrs* as a target pAU20*rrn* gene for qPCR, and used the primer pair 5′-AACGCGCAGAACCTTACCAG-3′ and 5′-TGCGGGACTTAACCCAACAT-3′ to amplify a 133-bp product. The real-time qPCR assay was performed in a 20-μL reaction mixture (FastStart Essential DNA GreenMaster, version 02; Roche), supplemented with the EcoRI-digested total DNA (0.2 ng) as a template and the above primer pair, by using LightCycler Nano (Roche). The cycling conditions consisted of an initial 10 min at 95 °C, followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 15 s. A melting curve analysis was then conducted from 60–95 °C at 0.1 °C·s^{-1}. Cycle threshold values were determined with the second derivative maximum method by using a LightCycler Nano SW 1.0 (Roche). Standard curves to calibrate the numbers of *rpsB* and *rrs* molecules with the threshold cycle numbers were generated by conducting qPCR assays with serial 10-fold dilutions of the respective DNA fragments that had been amplified by PCR using the same primer sets as above. The copy number of pAU20*rrn* per chromosome equivalent was calculated as the molecular ratio of *rrs* to *rpsB*.

Draft Genome Sequencing. A paired-end genome library was constructed from total DNA and sequenced by using MiSeq (Illumina). Obtained reads were assembled by using Newbler, version 2.6. The resulting contigs were examined for their possible linkage by using GenoFinisher and AceFileViewer. When necessary, PCR was performed using total DNA as a template and the primers designed from the contigs to confirm the actual linkage. The genome sequences were annotated by using MiGAP. Putative *oriC* or *dif* sites were located by using BLAST searches for *hemE* and *maf* homologs or for a 28-nt *dif*-like motif, respectively, using the AU20 sequence as a query.

Phylogenetic Analysis. Nucleotide sequences (Table S5) or amino acid sequences were aligned, and the maximum likelihood trees were built according to the Tamura–Nei model or the Jones–Taylor–Thornton matrix-based model, respectively, by using MEGA version 5.2 (36). For phylogenetic analyses based on the 16S rRNA gene, we used sequences corresponding to nucleotide numbers 109–1,406 of the *E. coli* gene. Multilocus sequencing analysis was performed by using five genes: *gyrB*, *rpoB*, *rpoC*, *atpD*, and *dnaK*. The sequence of each orthologous gene was trimmed to a uniform length in strains (2,372 bp for *gyrB*, 4,131 bp for *rpoC*, 4,152 bp for *rpoB*, 988 bp for *recA*, and 1,805 bp for *dnaK*). The five sequences from each strain were concatenated before being aligned. To confirm the tree topology, 1,000 bootstrap trials were performed.

Database Survey for RLC. On November 30, 2014, there were 3,150 RNA gene table (rnt) files, showing replicons carrying rRNA and/or tRNA genes, from 2,767 bacterial strains with complete genome sequences in the NCBI database. We regarded those replicons as including the main chromosomes of the respective strains, from which we selected 228 replicons as lacking rRNA genes. We subsequently selected three replicons, each of which was the largest (i.e., the main chromosome) in each strain. The absence of rRNA genes (or their remnants) was confirmed by RNAmmer (37) and nucleotide-nucleotide BLAST (blastn) searches.

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