

Genome Organization of *Pseudomonas stutzeri* and Resulting Taxonomic and Evolutionary Considerations

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In order to determine the genome variability within *Pseudomonas stutzeri*, 20 strains representing the seven described genomovars and strain JM300 were analyzed by using various resolution levels of rare cutting enzymes. *Xba*I and *Spe*I fingerprints revealed a high degree of heterogeneity of restriction patterns that did not correlate with the division into genomovars. However, a fragment pattern comparison led to the establishment of several groups of clonal variants within genomovars. One circular chromosome ranging in size from 3.75 to 4.64 Mb constitutes the genome of *P. stutzeri* strains. The I-*Ceu*I, *Pac*I, and *Swa*I low-resolution map of *P. stutzeri* type strain CCUG 11256 shows the locations of 12 genes, including *rrn* operons and the origin of replication. I-*Ceu*I digests of the 20 strains studied plus the positions of six genes allowed a comparison of the *rrn* backbone organization within genomovars; the four *rrn* operons seemed to be at similar locations with respect to the origin of replication, as did the rest of the genes. However, a comparison of I-*Ceu*I cleavage maps of the genomovar reference strains revealed a diverse genome organization in the genomovars relative to *rrn* operons and gene locations. In most genomovars, *rrn* operons are not arranged around the origin of replication but are equally distributed on the chromosome. Strain JM300 does not belong to any described genomovar, as determined from the organization of its genome. Large chromosomal rearrangements seem to be responsible for the differences in superordinate genome structure and must have played an important role in *P. stutzeri* diversification and niche colonization. An ancestral chromosome is suggested, and some plausible pathways for the generation of the various genome structures are proposed.

Pseudomonas stutzeri is a widely distributed nonfluorescent *Pseudomonas* species. This organism is easy to distinguish from other pseudomonad species by its ability to use maltose and starch as sole carbon and energy sources and its production of dinitrogen gas from nitrate. Workers have become interested in this species because of its metabolic properties, such as degradation of environmental pollutants (2, 73) and high-molecular-weight polyethylene glycols (55). *P. stutzeri* ZoBell and JM300 have been used as model microorganisms to study denitrification (17, 84) and natural transformation (50) processes in detail. The unique amylase produced by *P. stutzeri* has been the subject of biochemical studies because it hydrolyzes starch exoglycolytically but releases α -anomeric oligosaccharides (22). A variety of human infections have also been associated with *P. stutzeri* (32).

P. stutzeri is a member of the genus *Pseudomonas sensu stricto* in the gamma subclass of the *Proteobacteria*. DNA-DNA hybridization studies have defined seven clear-cut genomic groups called genomovars (68, 81). However, the *P. stutzeri* genomovars are characterized by a high level of heterogeneity of phenotypic properties (68, 69, 72), which does not allow the taxa to be split into different species. The phylogenetic relationships of 14 strains representing the seven described genomovars were determined recently by workers who compared 16S rRNA sequences, and the results confirmed the genomovar division (4). The 16S rRNA sequence of genomovar 6

strains was found to be different enough to rename this genomic group a new species, *Pseudomonas balearica*.

Information on genome structure is a very important component of any comprehensive description of a microorganism (57), and the potential applications of this information to bacterial systematics have been emphasized recently (11, 57, 66). Comparative analysis of bacterial chromosomes at the intra- and interspecies levels can provide information about genomic diversity, phylogenetic relationships, and chromosome dynamics. Intraspecies map comparisons have been performed with *Escherichia coli* (5, 58), *Leptospira interrogans* (83), *Mycoplasma hominis* (39), *Mycoplasma mycoides* (59), *Bacillus cereus* (9), *Clostridium perfringens* (7), *Rhodobacter capsulatus* (19), *Pseudomonas aeruginosa* (75), *Salmonella enterica* (42, 43, 45, 47), *Helicobacter pylori* (35), and *Lactococcus lactis* (41).

I-*Ceu*I is an intron-encoded endonuclease (23) that specifically cuts a 19-bp recognition sequence (53) within the *rrl* gene of a wide variety of bacteria (24, 33, 44, 56, 63). Since the number and locations of rRNA genes which can be considered the backbone of a chromosome are thought to be conserved in the chromosomes of bacterial species (18), I-*Ceu*I digests should be useful for analyzing chromosome structure. So far, comparisons of I-*Ceu*I digests of chromosomes of related species have been used as a tool in comparative studies and to detect DNA rearrangements (42, 44, 46, 48, 80).

Our knowledge concerning *Pseudomonas* genomes is limited to three species, *P. aeruginosa* (31, 64, 75), *Pseudomonas fluorescens* (60), and *Pseudomonas putida* (54). Genome fingerprinting was recently used to estimate the genome sizes of 16 *P. stutzeri* strains (61). However, no restriction map of this organism has been published. In this report we describe the basic genome organization of *P. stutzeri*. An analysis of *Xba*I and *Spe*I fingerprints allowed us to characterize bacterial strains on the basis of their unique restriction patterns and to identify several groups of clonal variants among the strains

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TABLE 1. *P. stutzeri* strains used in this study

Strain ^a	Genomovar	Source	Other designation(s)
ATCC 17589	1	Clinical isolate (Copenhagen, Denmark, before 1966)	222 ^b
CCUG 11256 ^{Tc}	1	Clinical isolate (Copenhagen, Denmark, before 1966)	ATCC 17588 ^T , 221 ^{Tb}
B1SMN1	1	Wastewater (Menorca, Spain, 1988)	
B2SMN1	1	Wastewater (Menorca, Spain, 1988)	
S1MN1	1	Wastewater (Menorca, Spain, 1988)	
ATCC 17591 ^c	2	Clinical isolate (Copenhagen, Denmark, 1956)	224 ^b
ATCC 17587	2	Clinical isolate (Copenhagen, Denmark, 1956)	220 ^b
ZoBell	2	Marine isolate (before 1944)	ATCC 14405
DSM 50227 ^c	3	Clinical isolate	ATCC 11607
LSMN2	3	Marine isolate (Barcelona, Spain, 1988)	
AN10	3	Marine isolate (Barcelona, Spain, 1982)	
AN11	3	Marine isolate (Barcelona, Spain, 1982)	
ST27MN2	3	Marine isolate (Barcelona, Spain, 1988)	
CH88	3	Water isolate (Mendoza, Argentina, before 1970)	
19SMN4 ^c	4	Marine isolate (Barcelona, Spain, 1988)	DSM 6084
ST27MN3	4	Marine isolate (Barcelona, Spain, 1988)	
DNSP21 ^c	5	Wastewater (Mallorca, Spain, 1988)	DSM 6082
SP1402 ^{Tc}	6 ^d	Wastewater (Mallorca, Spain, 1988)	DSM 6083
LS401	6 ^d	Marine isolate (Barcelona, Spain, 1988)	
DSM 50238 ^c	7	Soil isolate (Berkeley, Calif., before 1966)	ATCC 17832, 419 ^b
JM300		Soil isolate (California, before 1982)	

^a Strains were isolated or received under these designations. ATCC, American Type Culture Collection, Rockville, Md.; CCUG, Culture Collection of the University of Göteborg, Göteborg, Sweden; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

^b Strain designation assigned by Stanier et al. (77a).

^c Genomovar reference strain (68).

^d Genomovar renamed *P. balearica* sp. nov. (4).

examined. We present I-*CeuI* physical maps of the genomovar reference strains and a *SwaI*-I-*CeuI*-*PacI* low-resolution physical map of the *P. stutzeri* type strain. An I-*CeuI* cleavage map of *P. stutzeri* JM300 was also constructed. Various genes were located on the maps. Our results and map comparisons led to the following observations: (i) strains belonging to the same genomovar seem to have similar genome architectures; (ii) diverse genome organizations occur in the *P. stutzeri* genomovars; (iii) the rRNA genes are not concentrated around the origin of replication in most genomovars; (iv) strain JM300 does not belong to any described genomovar, as determined from its genome organization; (v) there is a correlation between taxonomic and phylogenetic data and genome structures; and (vi) DNA rearrangements may have played an important role in the diversification and evolution of *P. stutzeri*.

MATERIALS AND METHODS

Bacterial strains. A total of 20 strains representing the seven described genomovars (68, 71) were used in this study (Table 1). Genomovars 4 through 7 were established with less than three strains. In the case of genomovars 1 through 3, three to six strains were chosen by considering time, source, and geographical area of isolation. *P. stutzeri* JM300 (8), which was not included in any of our previous taxonomic studies, was also used in this study in order to validate our conclusions.

Plasmid detection in *P. stutzeri*. Plasmids were detected by the alkaline lysis procedure (74), the Eckhardt method (16), and pulsed-field gel electrophoresis (PFGE) of undigested DNA plugs. Agarose gels (0.8 and 0.6% agarose) were used for conventional electrophoresis. The Eckhardt method was applied to both conventional and PFGE gels.

Preparation of high-molecular-weight DNA. Bacterial strains were grown overnight at 30°C in Luria broth. After centrifugation, cells were resuspended at a concentration of 4×10^9 cells per ml. The method used to prepare DNA has been described previously (67).

Digestion of DNA in agarose blocks. Prior to digestion, agarose plugs were equilibrated three times with restriction buffer. Single digestions were performed as described previously (67) for *SpeI*, *XbaI*, and *PacI*. To improve the efficiency of I-*CeuI* digestion, one-half of a plug was digested for 3 h at 37°C with 3 U of enzyme in a 65- μ l reaction mixture. *SwaI* restrictions were done at 25°C for only 2.5 h. Double digestions were performed sequentially. I-*CeuI* partial digests were obtained after digestion with 3 U of enzyme for 40 min. I-*CeuI*-, *PacI*-, and *SwaI*-digested blocks were treated with 1 ml of ES (0.5 M EDTA, 1% [wt/vol]

N-lauroylsarcosine, 0.5 mg of proteinase K per ml [pH 9.5]) at 56°C for 45 min and then equilibrated with 1 ml of TE buffer (10 mM Tris, 10 mM EDTA [pH 7.4]). *XbaI* and *SpeI* digestions were stopped by adding 1 ml of TE buffer at 4°C. DNA end labelling was carried out as described previously (67). Restriction endonucleases were purchased from New England Biolabs (I-*CeuI*, *XbaI*, *SpeI*, and *PacI*) and Boehringer Mannheim (*SwaI*).

Fragment nomenclature. DNA fragments produced by single restriction endonuclease digestion were designated Pa (*PacI*), Sw (*SwaI*), and Ce (I-*CeuI*) to identify the enzyme, followed by capital letters (A, B, C, etc.) in order of decreasing fragment size. Fragments produced by double digestion were assigned numbers (1, 2, 3, etc.) in the same way.

PFGE. PFGE was performed in a contour-clamped homogeneous electric field (CHEF) model DRII and DRIII apparatus (Bio-Rad), in a Gene Navigator apparatus (Pharmacia LKB), or in a purpose-built crossed-field gel electrophoresis unit (77); 0.25 \times TBE buffer was used for the crossed-field gel electrophoresis apparatus, and 0.5 \times TBE buffer was used for the other apparatuses. Agarose concentrations and running conditions were varied in order to obtain optimal resolution of the different molecular weight ranges. *BstEII*-digested lambda DNA, lambda oligomers, *Saccharomyces cerevisiae* X2180-1B, *Candida albicans* LBS562, and *Schizosaccharomyces pombe* DSM 70576 were used as size standards. Two-dimensional gels were prepared by methods reported earlier (65).

Plasmids and gene probes. The origins of the *catA* (catechol-1,2-dioxygenase), *oprF* (outer membrane protein F), *aceAB* (pyruvate dehydrogenase), *recA* (RecA protein), and *hemL* (glutamate-1-semialdehyde-2,1-aminomutase) genes have been described previously (31, 34). *ori* (origin of replication) and *citA* (citrate synthase) probes were obtained by PCR amplification from *P. aeruginosa* as described previously (15, 82) and were kindly provided by C. Spangenberg. *ssb* (single-strand binding protein) and *hemB* (5-aminolevulinic acid dehydratase) *P. aeruginosa* genes were kindly provided by J. Genschel (23a) and D. Jahn (34a), respectively. The freeze-squeeze method (79) was used to elute the corresponding DNA bands from gels. Nonradioactive labelling of DNA fragments was carried out by random priming by using a Boehringer Mannheim digoxigenin labelling kit. 16S rRNA, 23S rRNA, and *nosZ* (nitrous oxide reductase) labelled probes were obtained by directly introducing digoxigenin-dUTP during PCR amplification from the *P. stutzeri* type strain (16S rRNA, 23S rRNA) and *P. stutzeri* ZoBell (*nosZ*). The 16S rRNA and *nosZ* primers were kindly supplied by A. Bennisar and were designed according to published sequences (GenBank nucleic acid sequence database accession numbers U26262 and M22628, respectively). The 23S rRNA primers were selected by using data from reference 51.

Southern blots and DNA hybridizations. Pulsed-field gels were transferred to a nylon membrane by alkaline capillary blotting for 48 h. Hybridization was carried out as described previously (10) for 48 h. Signals were detected as recommended by the manufacturer (Boehringer Mannheim).

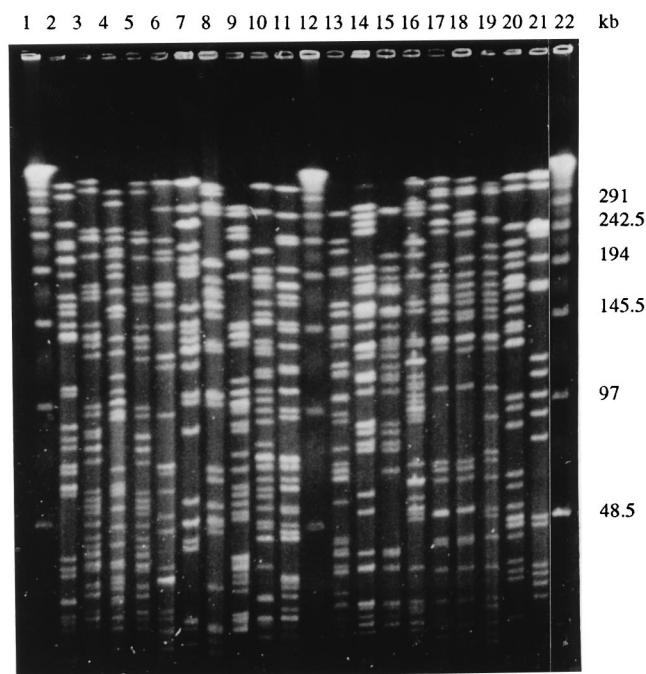


FIG. 1. Macrorestriction fragment patterns of chromosomes of *P. stutzeri* strains cut by *SpeI*. Lanes 1, 12, and 22, λ DNA concatemers; lane 2, DSM 50227; lane 3, ATCC 17587; lane 4, CCUG 11256^T; lane 5, ATCC 17591; lane 6, ATCC 17589; lane 7, DNSP21; lane 8, DSM 50238; lane 9, ZoBell; lane 10, 19SMN4; lane 11, ST27MN3; lane 13, AN11; lane 14, AN10; lane 15, ST27MN2; lane 16, LSMN2; lane 17, B1SMN1; lane 18, B2SMN1; lane 19, S1MN1; lane 20, SP1402; lane 21, LS401. The running conditions were as follows: 6.3 V/cm; reorientation angle, 120°; pulse times linearly increased in two ramps, 1 to 10 s for 15 h and 1 to 24 s for 23 h; agarose concentration, 1%; CHEF model DRIII apparatus.

RESULTS

Presence of plasmids in *P. stutzeri*. One to four plasmids were detected in 10 of the 21 strains analyzed (strains ATCC 17587, ATCC 17591, ATCC 17589, DNSP21, 19SMN4, ST27MN3, AN11, LSMN2, B1SMN1, and B2SMN2); 72% of the plasmids observed were smaller than 50 kb, one plasmid was between 50 and 95 kb long, and four plasmids were larger than 95 kb (strains 19SMN4, LSMN2, B1SMN1, and B2SMN2). No two strains shared the same plasmid profile. No relationship was found between the genomovars and the distribution of the plasmids among the strains. Interestingly, 7 of the 10 strains that contained plasmids were isolated from polluted environments. A correlation between the degree of contamination and the incidence of plasmids was found in the environmental studies of Baya et al. (3). The Eckhardt method used with both conventional and PFGE gels turned out to be the most reliable and useful method for plasmid detection.

***XbaI* and *SpeI* fingerprints and 16S rRNA and 23S rRNA restriction fragment length polymorphisms (RFLPs).** *XbaI* and *SpeI* restriction enzymes are known to cut the *P. stutzeri* genome into 24 to 42 fragments (61). Consequently, these enzymes were chosen to generate fingerprints in order to explore the *P. stutzeri* genome structure and to determine the degree of relatedness among the strains. The distribution and size range of the fragments generated with these two enzymes, especially *SpeI*, were optimal for PFGE resolution and for fragment pattern comparisons. The PFGE gels resolved 20 to 34 fragments in the case of the *SpeI* restriction patterns (Fig. 1) and 36 to 48 fragments in the case of the *XbaI* patterns. The similarity and degree of relatedness between macrorestriction

patterns were determined as described elsewhere (25). A unique restriction pattern characterized each strain. No correlation between the similarity of macrorestriction patterns and genomovar division was found due to the high discriminatory power of the two enzymes and the heterogeneity of the restriction patterns. A clear distinction between strains on the basis of G+C contents, which ranged from 60.9 to 65% mol% (68), was also not possible, as predicted by Rainey et al. (61). However, genomovar 2 strains (G+C contents, 60.9 to 62.3 mol% [68, 71]) could be distinguished on the basis of the absence of fragments larger than 175 kb in *XbaI* restriction patterns, as reported previously (61).

Strains ATCC 17591 and ATCC 17587, strains B1SMN1, B2SMN1, and S1MN1, and strains ST27MN2 and AN10 were identified as clonal variants from *XbaI* and *SpeI* fragment pattern comparisons. We believe that the strain 19SMN4-strain ST27MN3 pair is a borderline case that is not easy to define. In all cases, clonal variants were found within a genomovar. The common lineages reflected the origin and date of isolation, and the strains were phenotypically similar (68, 69, 72); exceptions are strains AN10 and ST27MN2, which were isolated in different years (1982 and 1988, respectively) from marine sediments in Barcelona, Spain.

Two *SpeI* sites (at positions 449 and 1096), one *I-CeuI* site (at position 1920), and no *XbaI* sites were found when the 23S rRNA sequence of *P. stutzeri* ZoBell was checked. Neither *XbaI* sites nor *SpeI* sites were found in the 16S rRNA sequence of *P. stutzeri* strain CCUG 11256. The 23S rRNA probe was designed to lie downstream of the two *SpeI* sites and the unique *I-CeuI* site. 23S rRNA and 16S rRNA RFLPs of *XbaI*- and *SpeI*-digested DNA revealed that there are four copies of both genes in the *P. stutzeri* genome. Identical patterns were obtained when *XbaI* fingerprints were hybridized with both probes, suggesting that the 16S rRNA and 23S rRNA genes are linked. One exception was found in the case of strain DNSP21 (genomovar 5), where one band smaller than the presumed operon size was detected in 16S rRNA RFLPs. In the case of strains belonging to a group of clonal variants, the probes recognized three or four bands with identical molecular weights; nevertheless, only one and two identical bands were recognized in *XbaI* RFLPs of strains AN10 and ST27MN2 and in *XbaI* RFLPs of strains B1SMN1, B2SMN1, and S1MN1, respectively.

Determination of genome parameters: size, number, and topology of the *P. stutzeri* chromosome. Chromosome size was determined by adding the *I-CeuI* restriction fragment sizes and by calculating the molecular weight of the linearized chromosome. The results are shown in Table 2.

The sizes of the chromosomes of the *P. stutzeri* strains ranged from 3.75 to 4.64 Mb. Differences in chromosome size of 70 to 427 kb were found among strains belonging to the same genomovar, while a maximum difference of 70 kb was found within clonal variants. The greatest range of variation was found with genomovars 3 and 1, the genomovars for which the most strains were analyzed. Differences in chromosome size of 0.9 Mb were found when genomovars were compared; however, there was no relationship between chromosome size and genomovars. The largest chromosomes were the chromosomes of genomovar 1 strains B1SMN1, B2SMN1, and S1MN1. The smallest chromosome, 3.75 Mb, was found for strain DSM 50238, the single member of genomovar 7. The average chromosome size was 4.29 Mb, and the standard deviation was 0.22 Mb.

A useful approach for establishing the size, topology, and multiplicity of a genome is separating the linearized chromosome. Plugs of undigested DNAs of all of the strains were

TABLE 2. Sizes of I-CeuI restriction fragments of *P. stutzeri* strains

Strain	Genomovar	Restriction fragment size (kb)				Chromosome size (Mb)
		CeA	CeB	CeC	CeD	
CCUG 11256 ^T	1	1,655	1,120	894	644	4.31 (4.35)
ATCC 17589	1	1,700	1,200	783	649	4.33
B1SMN1	1	1,800	1,060	1,060	726	4.64 (4.79)
B2SMN1	1	1,870	1,060	970	667	4.57
S1MN1	1	1,923	1,060	970	649	4.60 (4.90)
ATCC 17587	2	1,976	1,190	713	571	4.45 (4.53)
ATCC 17591	2	1,960	1,185	708	567	4.42 (4.62)
ZoBell	2	1,800	1,096	732	665	4.29 (4.48)
DSM 50227	3	1,660	928	873	730	4.19 (4.19)
AN11	3	1,560	1,060	1,006	668	4.29 (4.43)
AN10	3	1,750	1,144	955	650	4.50 (4.52)
ST27MN2	3	1,715	1,185	891	649	4.44 (4.55)
LSMN2	3	1,800	1,180	873	649	4.50 (4.66)
CH88	3	1,625	897	875	675	4.07
19SMN4	4	2,812	646	564	315	4.34 (4.55)
ST27MN3	4	2,625	758	667	218	4.27 (4.52)
DNSP21	5	1,675	1,006	720	649	4.05 (4.19)
SP1402	6	1,544	1,422	587	507	4.06 (4.12)
LS401	6	1,575	1,465	578	515	4.13 (4.02)
DSM 50238	7	1,338	936	820	660	3.75 (3.72)
JM300		1,630	1,130	1,009	263	4.03 (4.19)

^a Chromosome sizes are the sums of I-CeuI fragment sizes. The values in parentheses were obtained by comparing linearized chromosomes with *Schizosaccharomyces pombe* chromosomes.

electrophoresed under the optimal conditions for resolution of fragments in the megabase size range on a PFGE gel (Fig. 2). A single clear band was observed for almost all of the strains; nevertheless, in each case the brightest signal came from the DNA retained in the well, suggesting that a single circular chromosome constitutes the genome of *P. stutzeri*. When the resulting chromosome sizes were compared with the values obtained by adding the sizes of the I-CeuI fragments (Table 2), the greatest difference was 7%. The discrepancies were due to the inherent limitations of fragment size calculations (e.g., small separation distances) and the lack of markers with sufficiently exact molecular weights in the megabase size range.

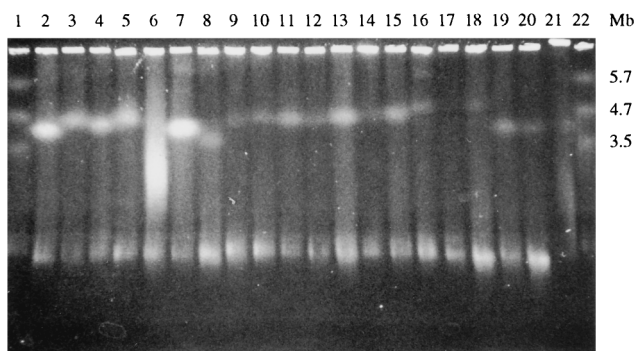


FIG. 2. Separation of undigested megabase pair replicons from *P. stutzeri* strains. Lanes 1 and 22, *Schizosaccharomyces pombe*; lane 2, DSM 50227; lane 3, ATCC 17587; lane 4, CCUG 11256^T; lane 5, ATCC 17591; lane 6, ATCC 17589; lane 7, DNSP21; lane 8, DSM 50238; lane 9, ZoBell; lane 10, 19SMN4; lane 11, ST27MN3; lane 12, AN11; lane 13, AN10; lane 14, ST27MN2; lane 15, LSMN2; lane 16, B1SMN1; lane 17, B2SMN1; lane 18, S1MN1; lane 19, SP1402; lane 20, LS401; lane 21, JM300. The separation conditions were as follows: linear pulse ramp from 2,000 to 4,500 s for 140 h at 1.3 V/cm in a 0.6% agarose gel. A CHEF model DR11 apparatus was used.

Genome architecture of *P. stutzeri*: I-CeuI skeleton and locations of 16S rRNA, 23S rRNA, *ori*, *recA*, *nosZ*, and *catA* genes. I-CeuI digests of the 20 strains yielded four fragments, indicating that four copies of the *rrl* gene are present in the *P. stutzeri* chromosome (Table 2 and Fig. 3), which was consistent with the results of the 23S rRNA and 16S rRNA fingerprints of *Xba*I- and *Spe*I-digested DNA. Consequently, the I-CeuI site is present in all *P. stutzeri* *rrl* genes and is not found in other parts of the chromosome or protected from digestion.

Similar I-CeuI patterns were observed in the same genomovar, with small size variations for the four fragments (Table 2 and Fig. 3). In strains B1SMN1, ATCC 17589, AN11, and DSM 50227 (genomovars 1 and 3), more pronounced deviations in size were observed for more than one fragment. When the patterns of the different genomovars were compared, a similar fragment size distribution seemed to be common to genomovars 1, 2, 3, and 5. Very different patterns were found for genomovars 4, 6, and 7, indicating that rRNA operon locations were not conserved within the species.

A limited set of housekeeping genes is used to define the backbone of the chromosome in bacteria (11). 16S rRNA, 23S rRNA, *ori*, and *recA* genes were localized on the I-CeuI fragments by hybridization. *nosZ* and *catA* were also mapped as genes that are present in *P. stutzeri*. Hybridization patterns were conserved within genomovars. When the hybridization patterns of the genomovars were compared, the origin of replication was always located on the biggest fragment (fragment CeA). However, different hybridization patterns were obtained with 16S rRNA, 23S rRNA, *recA*, *nosZ*, and *catA* probes for genomovars 2, 4, 6, and 7, while genomovars 1, 3, and 5 exhibited conserved hybridization patterns with these gene probes.

Intraspecies comparative mapping: low-resolution physical map of type strain CCUG 11256^T with gene locations and I-CeuI cleavage maps of genomovar reference strains. To construct a low-resolution physical map, restriction enzymes that cut the DNA into 5 to 15 fragments were sought. The G+C content of *P. stutzeri* CCUG 11256^T (T = type strain) has been reported to be 65 mol% (68). Consequently, a total of 22 restriction enzymes, including enzymes that harbor AT-rich recognition sites of 6 or 8 bp or the rare tetranucleotide CATG, were tested. The majority of these enzymes cleaved the DNA into more than 100 fragments. *Swa*I, *Pac*I, and I-CeuI generated one, four, and four fragments, respectively, and were chosen for map construction. Single and double digests were characterized (Tables 3 and 4), which resulted in an average chromosome size of 4,336 kb. A complete circular physical map was established from a single I-CeuI-*Pac*I two-dimensional gel and data from double and single digests (Fig. 5). Hybridization of 16S rRNA, 23S rRNA, *nosZ*, *catA*, *ori*, *recA*, *ssb*, *hemL*, *hemB*, *oprF*, and *citA* gene probes to fragments from single and double digests confirmed the established order of fragments and generated the data for a preliminary genetic map (Fig. 5).

Since differences in I-CeuI digests and gene hybridization patterns among *P. stutzeri* genomovars were found, I-CeuI cleavage maps were constructed in order to clarify the apparent variability of genome architecture. As determined from the conservation of the I-CeuI patterns and gene locations within genomovars, I-CeuI cleavage maps of the genomovar reference strains provided a representative model for each genomic group and revealed the diversity of genomic organization within the species. Because the order of only four fragments had to be determined, a partial-digest approach was chosen to construct the physical maps (Fig. 4). After partial I-CeuI digestion the fragments were hybridized with either *catA* or *recA*. Identification of partial-digestion fragments by using molecu-

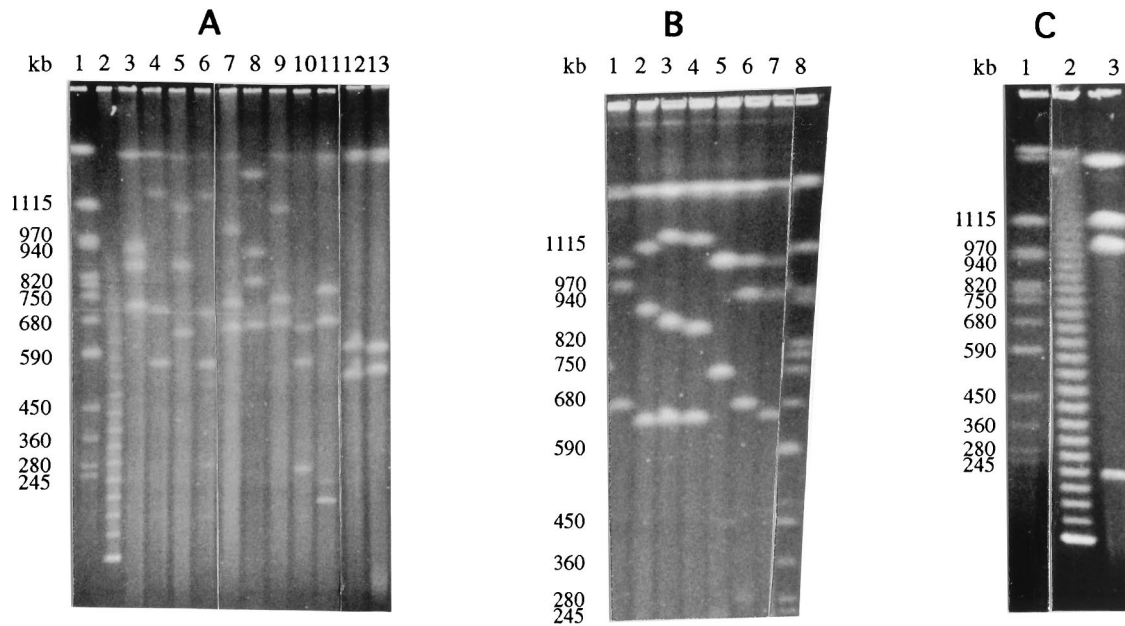


FIG. 3. I-*CeuI* digests of genomic DNAs from *P. stutzeri* strains. (A) Lane 1, *Saccharomyces cerevisiae*; lane 2, λ DNA concatemers; lane 3, DSM 50227; lane 4, ATCC 17587; lane 5, CCUG 11256^T; lane 6, ATCC 17591; lane 7, DNSP21; lane 8, DSM 50238; lane 9, ZoBell; lane 10, 19SMN4; lane 11, ST27MN3; lane 12, SP1402; lane 13, LS401. The running conditions were as follows: 6 V/cm; reorientation angle, 120°; pulse times, 40 to 150 s for 50 h; agarose concentration, 1.5%; CHEF model DR11 apparatus. (B) Lane 1, AN11; lane 2, AN10; lane 3, ST27MN2; lane 4, LSMN2; lane 5, B1SMN1; lane 6, B2SMN2; lane 7, S1MN1; lane 8, *Saccharomyces cerevisiae*; lane 2, λ DNA concatemers; lane 3, JM300. The running conditions were as follows: 3.3 V/cm; pulse times, 70 to 350 for 70 h; agarose concentration, 1%; CHEF model DR11 apparatus.

lar weight and gene probe signals resulted in seven I-*CeuI* cleavage maps, one for each genomovar reference strain (Fig. 5). Due to the ambiguous direction of the physical maps caused by the lack of a genetic map or other references, the *recA* gene was arbitrarily placed on the left side with respect to the origin of replication.

The positions of the rRNA genes clearly differ among the genomovars. In the case of strains CCUG 11256^T, ATCC 17591, DSM 50227, DNSP21, and DSM 50238 (genomovars 1, 2, 3, 5, and 7, respectively), rRNA genes are not clustered around the origin of replication and are almost equally distributed around the chromosome. In strain SP1402 (genomovar 6), three operons are located in a region that corresponds to 26% of the genome, and a fourth operon is on the opposite side of the chromosome. Two rRNA operons are thought to be on each side of *ori* in these strains. In strain 19SMN4 (genomovar 4), *rm* operons are grouped in a limited chromosome region (36% of the chromosome), but they are unequally distributed with respect to the origin of replication. There are no indications of differences in gene order in the low-resolution maps for the genomovar 1, 3, and 5 reference strains, but positions and gene order are remarkably altered in genomovars 2, 4, 6, and 7 compared with genomovars 1, 3, and 5, suggesting that several rearrangements have occurred. *sb* is at a different position in strains ATCC 17591 and 19SMN4. The *nosZ* and *catA* locations are altered in strain SP1402, while in addition *sb* also occupies a different position in strain DSM 50238.

***P. stutzeri* JM300: a new genomovar?** I-*CeuI* digestion of JM300 genomic DNA also yielded four fragments but generated a fragment size distribution different from the distributions observed with the seven genomovars. The positions of the 16S rRNA, 23S rRNA, *nosZ*, *catA*, *recA*, and *ori* genes were unique on the JM300 I-*CeuI* map (Fig. 5), indicating that the organization of the JM300 chromosome does not correspond

to the genomic model of any existing genomovar. Since distinction between genomovars on the basis of genome organization seems to be possible, JM300 could not belong to any of the previously described genomovars.

DISCUSSION

In this study we included a considerable number of strains which represent all of the genomovars of *P. stutzeri* and which had different sources and dates of isolation. These strains constitute the most extensively studied group of *P. stutzeri* strains in taxonomic and systematic terms (4, 68, 69, 71, 72).

Variation in chromosome size has been found in *P. stutzeri*, and the chromosome sizes range from 3.75 to 4.64 Mb. The *P. stutzeri* genome is smaller than the genome of its close relative *P. aeruginosa*, whose size has been reported to range from 5.2 to 7 Mb (75). The genome sizes of *P. stutzeri* are not consistent with values determined previously for some of the strains included in this study. Differences of up to 1.6 Mb have been found with sizes calculated by renaturation kinetics (14). Smaller differences (up to 670 kb) were found with values reported by Rainey et al. (61); underestimating genome size in this case was probably a consequence of the presence of doublets and triplets in the *SpeI* restriction patterns used for genome size estimates by these authors. Adding I-*CeuI* fragment sizes and determining the size of the whole chromosome are more reliable measures. We found length variations in all four I-*CeuI* fragments within genomovars which ranged between 8 and 288 kb. While DNA rearrangements can explain the variability, DNA segments between the four *rm* genes must also have been lost or gained. In this case, we expect that a mosaic-like structure would be found when a more detailed analysis of the *P. stutzeri* chromosome is performed. This view is supported by the high degree of heterogeneity of the *XbaI* and

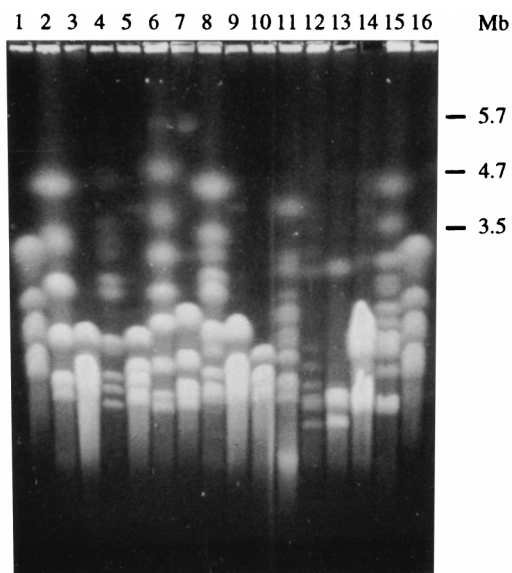


FIG. 4. I-CeuI complete and partial digests of DNAs of genomovar reference strains of *P. stutzeri*. Lanes 1 and 16, *Candida albicans*; lane 2, partial digest of DSM 50227 DNA; lane 3, complete digest of DSM 50227 DNA; lane 4, partial digest of CCUG 11256^T DNA; lane 5, complete digest of CCUG 11256^T DNA; lane 6, partial digest of ATCC 17591 DNA; lane 7, complete digest of ATCC 17591 DNA; lane 8, partial digest of DNSP21 DNA; lane 9, complete digest of DNSP21 DNA; lane 10, complete digest of DSM 50238 DNA; lane 11, partial digest of DSM 50238 DNA; lane 12, partial digest of 19SMN4 DNA; lane 13, complete digest of 19SMN4 DNA; lane 14, complete digest of SP1402 DNA; lane 15, partial digest of SP1402 DNA. The positions and sizes of the *Schizosaccharomyces pombe* chromosomes are indicated on the right. The running conditions were as follows: linear pulse ramp from 800 to 3,000 s for 140 h at 55 V in a 0.6% agarose gel. A Gene Navigator unit from Pharmacia LKB was used.

SpeI fingerprints which did not reflect the genomovar division. Evidence of mosaic structures has been found in *P. aeruginosa* (75). Natural transformation has been found in *P. stutzeri* (50), and plasmids are known to be common in this species (73; this study). Plasmids (or parts of plasmids) can be stabilized by integration into the *Pseudomonas* genome (29, 30, 76). Two genes of metabolic pathways which are thought to be related to lateral transfer (29, 84) have been found to be chromosomally encoded in *P. stutzeri*; *catA* codes for a catechol oxygenase I (38), a key enzyme in the metabolism of benzoate and other aromatic compounds, and *nosZ*, the nitrous oxide reductase gene (12), is a gene that has been implicated in nitrous oxide respiration. It has been proposed that incorporation of foreign DNA carried by plasmids into the chromosome of a common ancestor occurred during evolution of two species of the same genus, *P. putida* and *P. aeruginosa* (29, 30). Transposons have been detected in different *Pseudomonas* species (30, 76), al-

TABLE 3. Fragment sizes in single restriction enzyme digests of *P. stutzeri* CCUG 11256^T DNA

Fragment	Size (kb)		
	<i>PacI</i>	<i>SwaI</i>	I- <i>CeuI</i>
A	1,700	4,350	1,655
B	1,306		1,120
C	1,253		894
D	84		644
Total	4,343	4,350	4,313

TABLE 4. Fragment sizes in double restriction enzyme digests of *P. stutzeri* CCUG 11256^T DNA

Fragment	Size (kb)		
	<i>PacI</i> + I- <i>CeuI</i>	<i>SwaI</i> + I- <i>CeuI</i>	<i>PacI</i> + <i>SwaI</i>
1	1,226	1,655	1,394
2	1,092	1,120	1,306
3	859	890	1,253
4	610	339	306
5	456	306	84
6	48		
7	34		
8	33		
Total	4,358	4,310	4,343

though none has been described for *P. stutzeri*. We suggest that removal and addition of plasmid DNA, transposition, and natural transformation are mechanisms by which changes have occurred in the *P. stutzeri* genome and have generated part of the genotypic and phenotypic diversity exhibited by the genomovars and individual strains. Diversification by the mechanisms indicated above starts at the level of clonal variants. The differences in chromosome size and the small differences between the melting temperatures of homologous hybrids and the melting temperatures of heterologous hybrids (ΔT_m values) (0.2°C between strains 19SMN4 and ST27MN3; 0.0°C between ATCC 17587 and ATCC 17591) (68) make the hypothesis that point mutations are the first step of diversification unlikely.

Examples of the impact of "small-scale" genome rearrangements in *P. stutzeri* are strains ATCC 17589 and CCUG 11256^T of genomovar 1. The high degree of variability of restriction patterns cannot be related only to nucleotide divergence since a ΔT_m value of 0.0°C and an evolutionary distance of 0.3 have been reported for these two strains (4, 68). The 30-kb difference in chromosome size is not enough to explain acquisition or loss of *XbaI* and *SpeI* sites. Strain ATCC 17589 has a peculiar I-*CeuI* pattern within genomovar 1 and is the only exception to homogeneity of hybridization patterns. *recA* hybridized with fragment CeB in this strain, while this gene was localized in fragment CeC in the other strains of this genomovar. The RFLPs observed in *P. stutzeri* strains are a testimony to the genomic plasticity of this species. As proposed by Hall (26), DNA rearrangements may be responsible for a high proportion of the RFLPs used to differentiate and type bacteria.

In this paper we describe the construction of the first low-resolution physical map and a preliminary genetic map of *P. stutzeri* CCUG 11256^T. Also, I-*CeuI* cleavage maps of representative strains of the seven genomovars of *P. stutzeri*, as well as an I-*CeuI* cleavage map of strain JM300, were constructed. As in most prokaryotes, a unique circular chromosome constitutes the genome of *P. stutzeri*. A similar backbone organization of *rrn* operons was found within the genomovars, along with conserved locations of four genes. Strains belonging to a genomovar are defined by ΔT_m values of 2°C or less (68). These results suggest that there may be a correlation between genome organization and genetic relatedness. A comparison of I-*CeuI* cleavage maps revealed that the *rrn* backbone and gene order are not conserved among genomovars. The genome organizations of genomovars 1, 3, and 5 seem to be very similar within the low resolution of the maps. Genomovars 5 and 1 are the most closely related genomovars as determined by 16S rRNA data (4) and DNA-DNA hybridization data (68). The

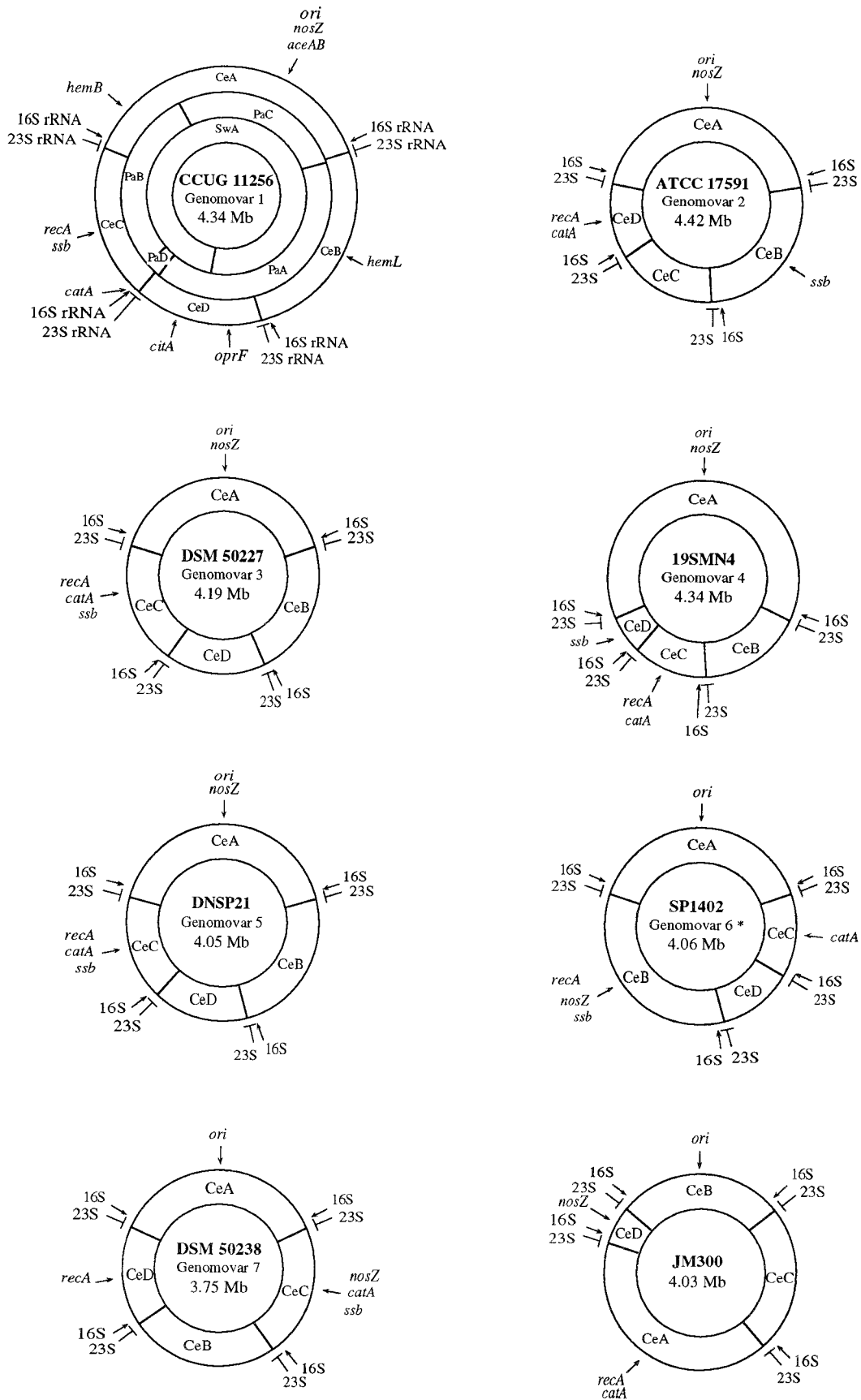


FIG. 5. Physical map of the chromosome of *P. stutzeri* type strain CCUG 11256 and I-*CeuI* cleavage maps of genomovar reference strains and strain JM300. Restriction sites for I-*CeuI*, *PacI*, and *SwaI* are indicated. The positions of mapped genes and *rrm* operons are shown on the circular maps. The positions of genes are indicated by arrows spanning the centers of the fragments where they are located. Genomovar 6 has been renamed *P. balearica*. Abbreviations: 16S, 16S rRNA gene; 23S, 23S rRNA gene.

chromosome structures of the genomovar 2, 4, 6, and 7 reference strains and strain JM300 are obviously distinct, as shown by rRNA operon locations and gene positions. The most different maps are the maps of strains SP1402 (genomovar 6), DSM 50238 (genomovar 7), and JM300. Genomovar 6 has been found to be the most distantly related genomovar by 16S rRNA sequence comparisons and has been designated a new species, *P. balearica* (4). Among the rest of the genomovars, genomovar 7 exhibited the greatest evolutionary distance (4). Strain DSM 50238 was also the most phenotypically and genotypically aberrant strain in the study of Rosselló et al. (68). We also propose that strain JM300 does not belong to any of the previously described genomovars. Phylogenetic and taxonomic data (70) are consistent with our results which show that a new genomovar (genomovar 8) should be created, with JM300 as the reference strain. In conclusion, it seems to be possible to distinguish the genomovars on the basis of genome organization. I-*CeuI* digests have been shown to be a powerful tool for analyzing gross genome structure and for comparative mapping. The taxonomic criteria provided by map comparisons resulted in conclusions consistent with the division of *P. stutzeri* into seven genomovars. We think that the results which we obtained, even though our maps are rudimentary, should have significance for understanding chromosome structure and genome evolution and should confirm that bacterial genome structure has great potential in the field of bacterial taxonomy and systematics.

We demonstrated that I-*CeuI* cuts the *rrl* gene of *P. stutzeri* and no other sites. The number of rRNA operons is conserved within *P. stutzeri*, but the locations are not conserved, in contrast to *Salmonella enterica* (43, 45, 48) and *P. aeruginosa* (75). As in *P. aeruginosa* (75), four *rrn* operons are present in all strains, but these operons occur at different positions with respect to the origin of replication. In genomovars 1, 2, 3, 5, and 7, rRNA operons are equally distributed around the chromosome. To date, this arrangement does not seem to be common among procaryotes. In the species that have been analyzed, the *rrn* operons are grouped in a region corresponding to 37 to 52% of the chromosome around *ori* (41). In genomovar 4, the *rrn* operons are grouped in a limited chromosome region (36% of the chromosome) but are distant from the origin of replication. However, in strain JM300 the rRNA operons cluster in a region corresponding to 59% of the genome around *ori*. In all of the genomovars except genomovar 4, the *rrn* operons are distributed in two groups of two operons on each side of *ori*, in contrast to most procaryotes, in which the *rrn* operons are unequally distributed (11). All of the *rrn* operons supposedly are transcribed divergently away from *ori*.

In bacteria, the origin of replication is situated almost diametrically opposite the terminus of replication (11), resulting in two replication arms of similar lengths. rRNA operons are generally transcribed divergently away from the origin of replication (11) and are oriented in such a way that transcription occurs in the same direction as replication (6). If the same arrangement occurs in *P. stutzeri*, the terminus of replication should be situated in the region delimited by the two rRNA operons with inverted orientations (fragment CeD in strains CCUG 11256^T, DSM 50227, DNSP21, and SP1402; fragment CeB in strains 19SMN4 and DSM 50238; and fragment CeC in strain ATCC 17591).

Different kinds of genetic events (deletions, insertions, translocations) may have played a role in the generation of the different genome structures of genomovars from an ancestor. However, gross genome changes, such as large inversions (recombinations) which restructure the whole chromosome, can explain for the most part the different genomic arrangements

found in the genomovars. As a working model, an ancestor chromosome which had genes and rRNA operons arranged in the order found in strains CCUG 11256^T, DSM 50227, and DNSP21 (genomovars 1, 3, and 5) is proposed (Fig. 6A and C). Strains CCUG 11256^T, DSM 50227, and DNSP21 have the same rRNA backbone, which may be similar to the situation before *P. stutzeri* branched into genomovars. The genome structures of all of the genomovars might be deduced from this ancestral chromosome. Below we outline possible patterns of evolution that could have yielded some of the different genome organizations. Genomovar 4 and genomovar 2 gross genome structures could have differed by a single large inversion between fragments CeA and CeB, including one rRNA operon, *ssb*, and the origin of replication (Fig. 6B). The symmetry of the origin of replication relative to the terminus would not have been highly altered according to this model; nevertheless, there is a displacement of genes outside the inverted segment relative to *ori*. Evolution of genomovar 4 from genomovar 2 would be consistent with the close relationship of these two genomovars determined by 16S rRNA analysis and DNA-DNA hybridization (4, 68). The changes observed in strains SP1402 and DSM 50238 (genomovars 6 and 7, respectively) and in strain JM300 must be more complex than a simple inversion. For example, genomovar 6 genome structure could have resulted from three consecutive inversions from the ancestor (Fig. 6C). A first large inversion, between fragments CeC and CeB of the ancestor, would have placed *recA* and *ssb* on the opposite side of the chromosome. A second large inversion, between the generated CeA' and CeB' fragments, including one rRNA operon, the origin of replication, *ssb*, and *recA*, would have yielded fragments CeA and CeB of SP1402. A third inversion, between fragments CeC' and CeD', would have shifted the *catA* location and the rRNA operon orientation, yielding fragments CeD and CeC from SP1402. This third change would have been initiated by the significant asymmetry of the origin of replication relative to the terminus caused by the second inversion, and it would have implicated the terminus of replication resembling inversions found in *Salmonella typhi* Ty2 (47) and *E. coli* K-12 (58). Inversions proposed for genome evolution of strain SP1402 (genomovar 6) affect large regions of the genome and would greatly alter gene dosage and symmetry of the origin with respect to the terminus of replication. In general, it has been thought that the rate of occurrence of inversions is low compared with other kind of rearrangements, but studies have been limited to a few species and some of the species used are known to live in a restricted environment, which might explain their remarkable evolutionary stability (43, 45, 62). However, there is increasing evidence from recent map comparisons that chromosomal inversions might shape the genomes of species or subspecies. Fossilized inversions have been characterized recently (19, 41, 42, 45, 47, 62), and evidence of such rearrangements has also been found in other microorganisms (9, 13, 39, 49, 59, 83), indicating that inversions are not seldom established in natural populations. Natural inversions are not necessarily consistent with the rules that control *E. coli* or *Salmonella typhimurium* inversions under laboratory conditions (37, 52). Hill et al. (28) and François et al. (21) easily obtained revertants from inverted chromosomes when bacteria were grown rapidly in rich media. But this situation is probably unusual for many microorganisms which have to adapt to different, harsh environmental conditions and usually have low rates of growth in nature. Growth rate alone, as determined in the laboratory, could never be the only factor that determines survival and successful spread in natural environments. Interestingly, all naturally occurring inversions that have been described, such as those thought to generate the

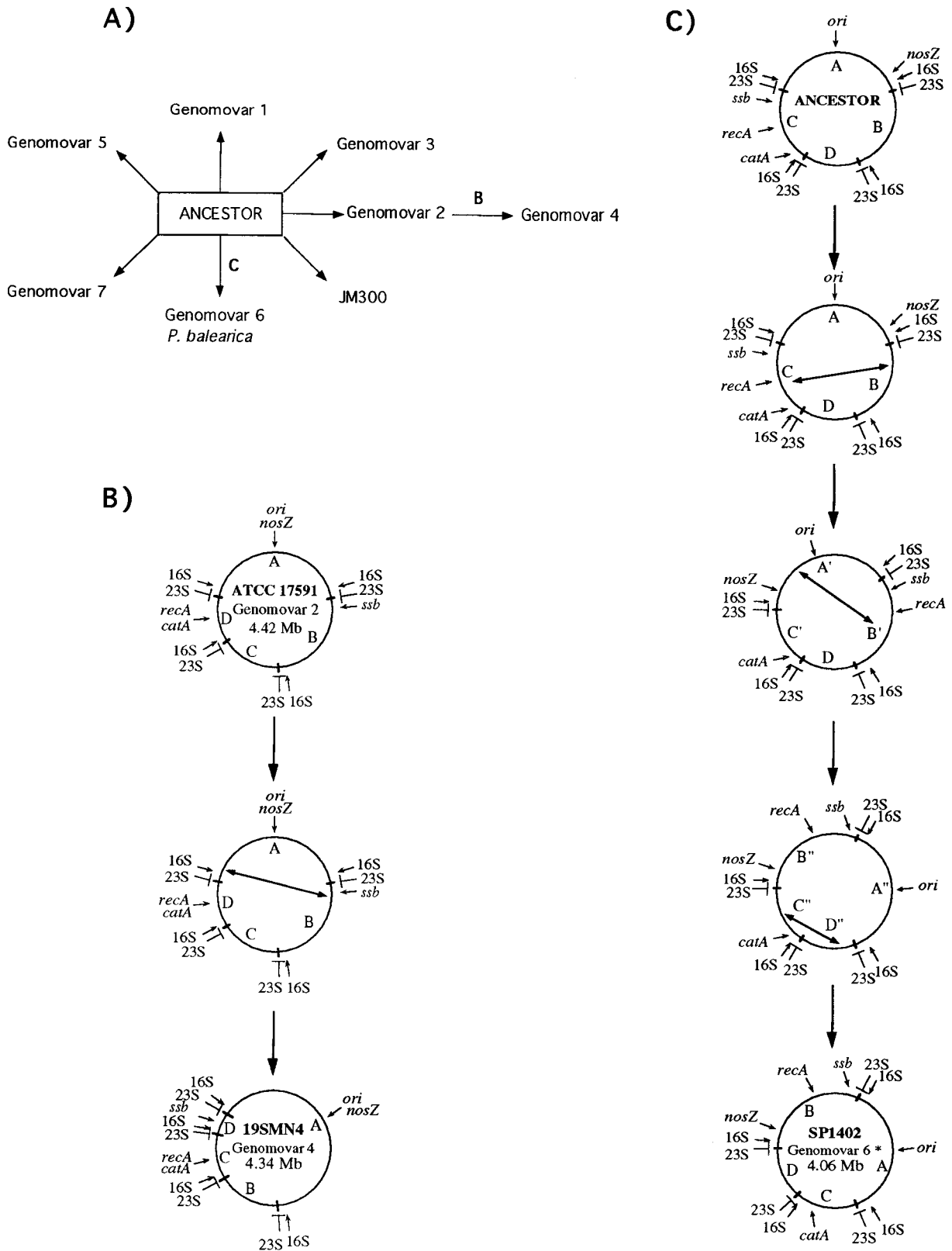


FIG. 6. (A) Generation of *P. stutzeri* genomovars: *P. stutzeri* diversification from a common ancestor on different lines of evolution. The ancestor would have I-*CeuI* fragments and genes arranged in the order found in strains CCUG 11256^T (genomovar 1), DNSP21 (genomovar 5), and DSM 50227 (genomovar 3). (B) Possible evolutionary pathway for creating genomovar 4 genome structure from genomovar 2. (C) Possible evolutionary pathway for creating genomovar 6 (*P. balearica*) genome structure from the ancestor (for explanation, see the text). Abbreviations: 16S, 16S rRNA gene; 23S, 23S rRNA gene.

chromosome structures of genomovars 4 and 6, include the origin or the terminus of replication, and none is within a replication arm. These kinds of inversions should respect direction of transcription of genes. We do not know the rules that select inversions in natural populations; however, rearranged chromosomes must offer an advantage under environmental conditions, probably because of modifications to phenotypes (27, 28, 37) or modulations of gene expression (20). Inversions can also be responses of genomes to imbalances produced by other rearrangements, such as duplications, insertions, or deletions, or vice versa. Liu et al. proposed that inversions in *Salmonella typhi* and *Salmonella enteridis* could be caused by the 118- and 100-kb insertions in their respective genomes (45, 46). Cascades of rearrangements have been also reported for *Bacillus subtilis* (1). Bacterial genomes that undergo natural transformation like *P. stutzeri* could be more prone to such rearrangements, as has been proposed for *Campylobacter* strains (78). Homologous recombination between inverted repeated DNA sequences is a common mechanism for generating inversions. Inversions due to recombination between rRNA operons have been described (19, 28, 42, 46), but such inversions do not seem to have occurred during *P. stutzeri* evolution. Other recurrent chromosomal elements, such as insertion sequences (36), *t-RNA*, and *rhs*, or simple sequences were probably involved in generating the rearrangements suggested in this study.

Fonstein and Haselkorn (18) proposed that there are two levels of genome conservation in bacteria. Microorganisms with strict conservation of gene order, like *E. coli* (5, 58), *Salmonella enterica* (43, 45, 48), *Clostridium perfringens* (7), *Streptomyces* spp. (40), *Borrelia* spp. (56), *Haloflex* spp. (49), *Lactococcus lactis* (41), and *Mycoplasma* spp. (39, 59), form one group. Significant pressure must exist to maintain these stable chromosome structures. A second group includes microorganisms with highly rearranged chromosomes and no long-range conservation of genetic maps, such as *B. cereus* (9), *H. pylori* (35), and *R. capsulatus* (19). The large variation found among fingerprints and all of the genomovar maps indicates that genome organization is not conserved within *P. stutzeri* and that chromosome rearrangements are frequent in this species. For these reasons, we propose that *P. stutzeri* should be assigned to the second group of microorganisms. Considering the apparent plasticity of the *P. stutzeri* chromosome, we think that it would not be surprising to find more differences when more genes are localized. At least for one strain of genomovar 3, additional hybridization data revealed differences which suggested that large changes have occurred (data not shown).

Divergence within *P. stutzeri*, which is a widespread microorganism, may have occurred recently during colonization and adaptation to different environments or even microenvironments, as suggested recently by Schmidt et al. (75). Diversification of this species could be the result of niche-specific selection of (i) point mutations in genes, which cause gradually nucleotide divergence (considerable sequence divergence has occurred during evolution of *P. stutzeri* genomovars, as shown by G+C contents [68], ΔT_m values between 5.1 and 12.2°C [68], and evolutionary distances [4]), (ii) acquisition of new genes necessary to survive under environmental conditions by horizontal transfer of plasmids and transposons or via natural transformation that modifies the genetic content and size of the genome, (iii) short-range recombinations within genes that alter specificities, and (iv) large chromosome rearrangements that affect the three-dimensional structure of the nucleoid and probably its function. *P. stutzeri* evolution and diversification could be the result of independent genomic adaptations of an ancestor to different environmental pressures exerted by

niches which are difficult to trace. Environmentally adapted organisms, such as *P. stutzeri* and the human pathogenic bacterium *H. pylori* (35), which seems to exhibit unusual genome instability, should provide a novel view of the chromosome.

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