JOURNAL OF BACTERIOLOGY, Dec. 2000, p. 7014–7020 Vol. 182, No. 24
0021-9193/00/$04.00+0

pING Family of Conjugative Plasmids from the Extremely Thermophilic Archaeon *Sulfolobus islandicus*: Insights into Recombination and Conjugation in Crenarchaeota

KENNETH M. STEDMAN,[1]† QUNXIN SHE,[2] HIEN PHAN,[2] INGELORE HOLZ,[1] HARPREET SINGH,[1] DAVID PRANGISHVILI,[1]‡ ROGER GARRETT,[2] AND WOLFRAM ZILLIG[1]*

Max-Planck-Institut für Biochemie, D-82152 Martinsried, Germany,[1] and Institute of Molecular Biology, University of Copenhagen K, Sølvgade 83H, DK-1307 Copenhagen K, Denmark[2]

Received 17 April 2000/Accepted 21 September 2000

A novel family of conjugative plasmids from *Sulfolobus* comprising the active variants pING1, -4, and -6 and the functionally defective variants pING2 and -3, which require the help of an active variant for spreading, has been extensively characterized both functionally and molecularly. In view of the sparse similarity between bacterial and archaeal conjugation and the lack of a practical genetic system for *Sulfolobus*, we compared the functions and sequences of these variants and the previously described archaeal conjugative plasmid pNOB8 in order to identify open reading frames (ORFs) and DNA sequences that are involved in conjugative transfer and maintenance of these plasmids in *Sulfolobus*. The variants pING4 and -6 are reproducibly derived from pING1 in vivo by successive transpositions of an element from the *Sulfolobus* genome. The small defective but mobile variants pING2 and -3, which both lack a cluster of highly conserved ORFs probably involved in plasmid transfer, were shown to be formed in vivo by recombinative deletion of the larger part of the genomes of pING4 and pING6, respectively. The efficient occurrence of these recombination processes is further evidence for the striking plasticity of the *Sulfolobus* genome.

Conjugative plasmids (CPs) in archaea have so far been found only in closely related strains of the extremely thermophilic crenarchaeon *Sulfolobus*, where they occur frequently (8, 10). The complete DNA sequences of members of three subfamilies of these CPs, pNOB8 (13), pING (this paper), and pSOG2/4 (G. Erauso and J. van der Oost, personal communication), each contain only two open reading frames (ORFs) which show significant sequence similarity to genes essential for conjugation in bacteria. One shows low but significant similarity to *traG* (from plasmid RP4), and the other shows marginal similarity to *trbE* (13). The functions of both of these genes in bacterial conjugation remain somewhat obscure (6). Apart from these few insights gained from genome analysis, and from the observations that specific cell pairing precedes plasmid transfer (10) and that transfer is probably selective and unidirectional (8), little is known about the mechanism of archaeal conjugation. The genome of pNOB8, which in its natural host has a low copy number, has been shown to contain homologs of genes required for partitioning and maintenance of bacterial plasmids and chromosomes (13).

To assess the functional roles of putative gene products in conjugative transfer and maintenance of archaeal conjugative plasmids, in view of the absence of practical genetic methods for analyzing these genes in vivo, we determined the sequences of three variants of the pING family (pING1, pING4, and pING6) and present data on their gene composition. Moreover, we compare the ORFs in pNOB8 and the pING variants and consider their possible functional roles in archaeal conjugation.

The genomes of *Sulfolobus* CPs are rather stable during propagation by conjugative transfer and replication in the recipients. However, they vary dramatically when they are spread after electroporation and subsequently propagated in the recipients (8). The observation of the in vivo generation of complete and functionally defective pING variants and sequence comparison of these variants revealed novel and efficient mechanisms of genetic rearrangement in crenarchaeota, which we describe herein.

The two small plasmids pING2 and pING3 were unable to effect their own transfer but could be conjugationally transferred with the help of complete CPs. The comparison of their genomes with those of the complete CPs, and the recognition of a homologous conserved region in pNOB8, allowed us to distinguish between motifs and ORFs making these plasmids mobile for conjugative transfer and ORFs actively involved in transferring them. Here we report the first results of this functional dissection of conjugation in archaea.

MATERIALS AND METHODS

Detection and isolation of the pING plasmids. Methods for sampling, enrichment, plating, and colony cloning of *Sulfolobus* strains from solfataric fields in Iceland have been described previously (14, 15). All strains from the enrichment culture of the sample from a small mud pot on the north slope of mount Hengill with a maximal temperature of 95.3°C and a pH of 3 to 3.5 belonged to a species provisionally called *Solfolobus islandicus*, which is closely related to *S. solfataricus* (15). All strains from this source produced toxic proteins, sulfolobicins (9), but only one of them, HEN2P2, yielded covalently closed circular DNA (cccDNA) by the method of Birnboim and Doly (3). After introduction into *S. solfataricus* P1 by electroporation, this DNA spread through the culture and was amplified to high copy number, showing that it contained conjugative plasmids (8). The capacity to produce sulfolobicin was, however, not transferred. The plasmid mixture was dominated by one plasmid which yielded an *Eco*RI restriction fragment pattern later assigned to pING1. Pure strains containing the single CPs pING1, pING4, and pING6, each with a characteristic restriction pattern (Fig. 1), were isolated via colony plating of the culture obtained after electroporation and spreading on solid media.

* Corresponding author. Mailing address: Max Planck Institute for Biochemistry, Am Klopferspitz 18A, D-82152 Martinsried, Germany. Phone: 49-89-8578-2231. Fax: 49-89-8578-2728. E-mail: zillig@biochem.mpg.de.

† Present address: Thermal Biology Institute, Montana State University, Bozeman, MT 59717-3142.

‡ Present address: Department of Microbiology, University of Regensburg, 95053 Regensburg, Germany.

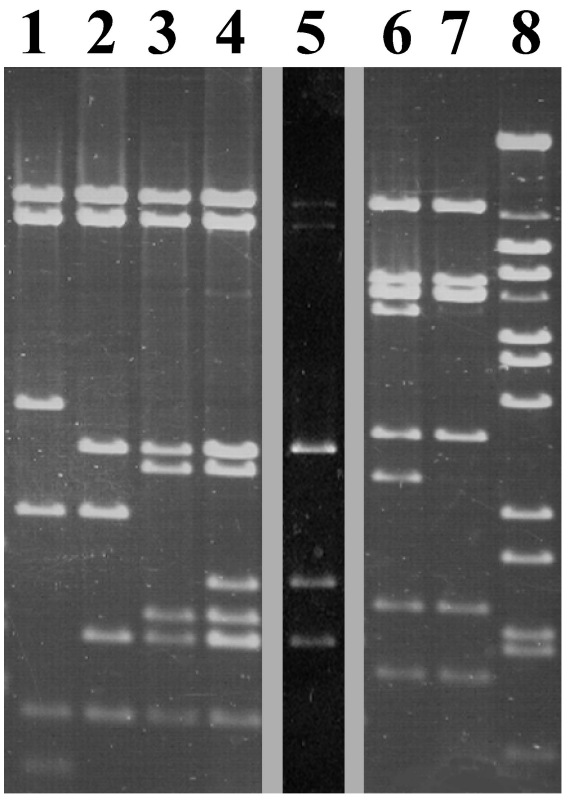

FIG. 1. *Eco*RI digestion patterns of plasmids pING1 (lane 1), pING4 (lane 2), pING6 (lane 3), a mixture of pING6 and pING3 (lane 4), pING3 (lane 5), pNOB8 (lane 6), and pNOB8-33 (lane 7). A DNA size standard (λ *Eco*91-I; 14.4, 8.5, 7.2, 6.4, 5.7, 4.8, 4.3, 3.7, 2.3, 1.9, 1.4, 1.3, and 0.7 kbp) is shown in lane 8.

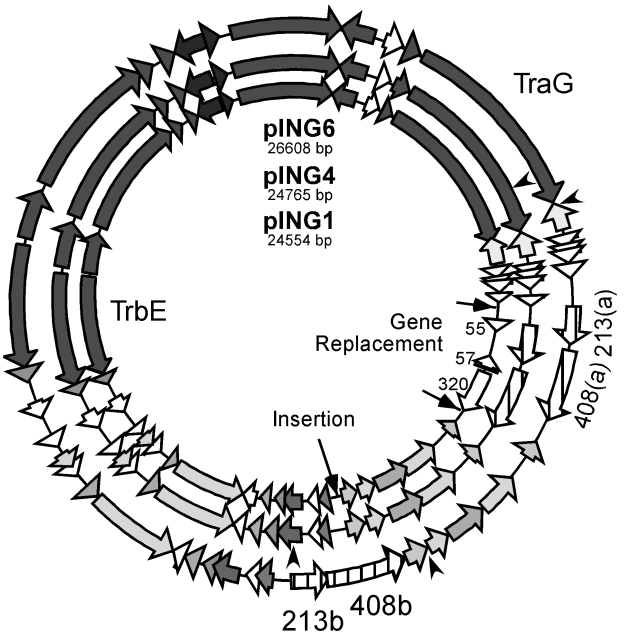

FIG. 2. Comparative ORF maps of pING6, pING4, and pING1, from outer to inner circle, respectively. The gene replacement in pING1 of ORF55, ORF57, and OR320 by tandem IS elements, ORF213a and ORF408a, is marked by two arrows inside the pING1 map. The precise insertion in pING4 of the same sequence (containing ORF213b and ORF408b), generating pING6, is shown by a single arrow inside the pING1 map. The ORFs in the tandems, numbered 213a, 213b, 408a, and 408b, are labeled with vertical stripes. The looping-out points of the functionally defective small plasmids pING2 from pING4 and pING3 from pING6 are marked by arrowheads next to the corresponding sequences.

Other *Sulfolobus* strains used were *S. solfataricus* P1 DSM1616, *S. acidocaldarius* DSM639, and various strains of *S. islandicus* isolated as described elsewhere (14, 15). The growth medium was as described previously (15). For liquid cultures 0.1% (wt/wt) Bacto Tryptone and 0.1% yeast extract were used as carbon sources; in Gelrite gels only 0.2% Bacto Tryptone was used; both were buffered with glycine to pH 3.5. Culturing and plating procedures and the conservation of strains have also been described elsewhere (15).

Purification of pING2 and pING3. Five hundred milliliters of an early-exponential-phase culture of *S. solfataricus* P1 (recipient culture) was inoculated, at an optical density at 600 nm (OD_{600}) of about 0.4, with 5 μl of 10-fold-concentrated transcipient stocks containing pING1, pING4, and pING6, respectively. These stocks were obtained by resuspending *S. solfataricus* P1, conjugated with donors of these plasmids, in pH 5 medium without carbon source and containing 20% (vol/vol) glycerol. After growth for about 2 days to an OD_{600} of 1.5, 10 ml of this culture was transferred to 500 ml of fresh medium and again grown to an OD_{600} of 1.5. The transfer into fresh medium and growth to an OD_{600} of 1.5 was repeated twice more in the same way. Plasmid DNAs were prepared according to Birnboim and Doly (3) after the primary conjugative transfer and after each of the following three growth phases.

The formation of pING4 from pING1, of pING2 from both of these, and of pING3 from pING6 in these transfer experiments was deduced from the changes of the *Eco*RI restriction patterns of the plasmid preparations after each step as described in Results. Because the defective but mobile small pINGs require the help of active CPs for transfer and spreading, transcipient clones containing only the defective CPs could not be obtained from single colonies. Therefore, these plasmids had to be purified from mixtures with their helpers. Since, however, in mixtures from late growth periods the small plasmids represented up to more than 90% of the total number of copies, they were isolated by micropreparative agarose gel electrophoresis, either directly or after linearizing the accompanying large plasmids with suitable restriction enzymes; pING3 was linearized with *Ava*I and *Sph*I, which cut pING6 but not pING3.

Curing. Curing was demonstrated by plating transcipient cultures after prolonged growth, counting large colonies, and showing that 10 out of 10 of these no longer contained plasmids and could be recipients again.

DNA isolation. DNA purification from *Sulfolobus* cells was performed by a variation of the procedure of Birnboim and Doly (3) as described previously (2).

DNA sequencing and genome analysis. DNA sequencing was performed as described by She et al. (13). All ORFs coding for more than 50 amino acids were found using the program Webtrans at the Virtual Genome Center (http://alces-.med.umn.edu). Almost all ORFs which overlapped by more than two to three codons were discarded. Potential ATG, GTG, and TTG start codons were found manually. Analyses of the *Sulfolobus* genome were made with the genome database (http://niji.imb.nrc.ca/sulfolobus/) (12). ORFs from pING were searched against the publicly available databases using BLAST (1) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Comparisons of ORFs in pNOB8 and pING were performed using the Genetics Computer Group suite of programs.

Nucleotide sequence accession number. Sequences of the pING plasmids have been submitted to GenBank under accession number AF233440.

RESULTS

Origin. Plasmid DNA isolated from clones of the sulfolobicin-producing *S. islandicus* isolate HEN2P2 (15; D. Prangishvili, personal communication) was a complex mixture of variants after electroporation into, and propagation in, *S. solfataricus* P1. However, one strain from a single colony of the P1 transcipient culture yielded predominantly, and in high copy number, a single cccDNA. It produced a characteristic *Eco*RI restriction pattern (Fig. 1) and was designated pING1.

CPs were propagated by electroporation or growth of transcipients until we learned that both methods produce extensive plasmid variation. In contrast, no variant formation occurred during multiplication by conjugation when we started with a small inoculum of the donor (1/1,000 to 1/10,000 of the recipient) (8). Plating of cultures containing the plasmid variants yielded transcipient colonies which produced strains carrying single plasmids, of which pING1, pING4, and pING6 (Fig. 1 and 2), each about 25 kb in size, were the most frequent. These larger plasmids were sometimes accompanied by smaller de-

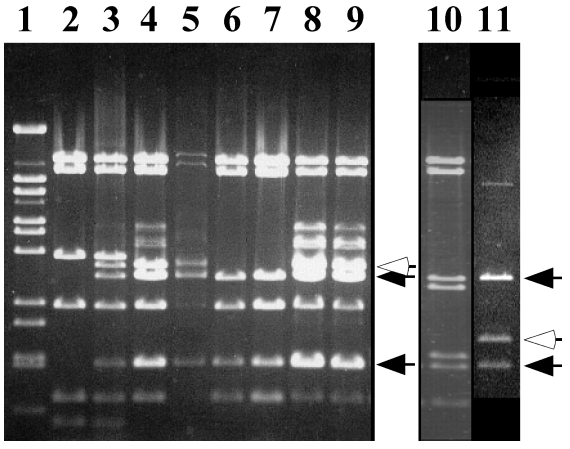

FIG. 3. *Eco*RI restriction digestion patterns of cccDNAs isolated after consecutive growth passages of transcipients of pING1 (lane 2) and pING4 (lane 6) showing the generation in vivo of pING4 from pING1 and of the small functionally defective CP pING2 from pING1 via pING4 (lanes 3 to 5) and from pING4 (lanes 7 to 9), respectively. Plasmid pING6 (lane 10) and its looped-out product pING3 (lane 11) are also shown for comparison. Invariant DNA fragments from pING2 and pING3 are indicated with filled arrows. The DNA fragments which differ between pING2 and pING3 are indicated with open arrows. A DNA size standard (λ *Eco*91-I; 14.4, 8.5, 7.2, 6.4, 5.7, 4.8, 4.3, 3.7, 2.3, 1.9, 1.4, 1.3, and 0.7 kbp) is shown in lane 1.

rivatives, predominantly pING2 and pING3, about 7 and 6 kb, respectively (Fig. 3 and 4), but also several others, all of which shared two *Eco*RI restriction fragments but differed in a third (Fig. 3). Upon conjugative transfer into *S. solfataricus* P1, both the large and the defective CPs were rapidly replicated to copy numbers of >50 per recipient chromosome. However, the recipients were cured of the plasmids during prolonged growth, indicating a subsequent blocking of replication. Moreover, the cured cells were temporarily resistant to conjugative transfer by these plasmids.

DNA sequence comparison. (i) ORFs. Of 38 ORFs in pING1 that are mapped in Fig. 5 and listed in Table 1, only 13 show no significant sequence similarity to ORFs in pNOB8 (13) (Fig. 5), even though some of these correspond in size, map position, and orientation to ORFs in pNOB8. Eleven ORFs, including ORF1042, a homolog of bacterial TraG proteins, and ORF640, a putative homolog of bacterial TrbE proteins, are highly conserved in both plasmids (Table 1). Amino acid sequence identities for these homologs in pING1 and pNOB8 range from 77.5 to 92.3% (Table 1) and, apart from an interruption by a small ORF which has no homolog in pNOB8, are contiguous.

Conversely, the genome of pING1 lacks homology to an uninterrupted cluster of 21 ORFs in pNOB8 flanked externally by the highly conserved ORF165 and ORF87 and to pNOB8 ORF620, ORF630a and ORF52 in a region discussed below (Fig. 5). Of these 21 ORFs, 13 are deleted as a result of a recombination between two flanking copies of a large repeat to yield the functional variant pNOB8-33 (13). Like pNOB8, pING1 is able to perform the basic functions of CP transfer, pair formation between donor and recipient cells, and plasmid transfer and replication in the transcipient. Therefore, pNOB8 ORFs that are absent from pING1 are not required for these basic functions. In its natural host, NOB8H1, pNOB8 is stably maintained at a low copy number and must therefore undergo copy number control and correct partitioning. On the other hand, the pINGs are replicated to very high copy numbers immediately after transfer and are lost during prolonged growth, showing a lack of control of copy number and partitioning. Thus, genes present in pNOB8 but not in the pINGs must be involved in these functions. Of the 21 contiguous ORFs in pNOB8 but not in pING1, one is homologous to ParA proteins and two are homologous to ParB proteins and are candidates for these functions.

(ii) IS elements. The large complete CP pING4 differs from pING1 in that it contains a tandem array of two insertion sequence (IS) elements containing ORF213 and ORF408 (Fig. 2), which have been inserted between two copies of the target site (TA)AGGG and replaced ORF55, ORF57, and ORF320 of pING1 (Fig. 5 and 6). An identical tandem array of these IS elements and a second, almost identical copy that is interrupted by a third type of IS element occur in the *S. solfataricus* P2 genome (Q. She and R. Garrett, unpublished data). The tandem appears to have been formed by joining two different IS elements with the same target site. The left target site in pING1, the left flanking target site duplicated in pING4, and the target site separating ORF213 and ORF408 are all perfect copies of the target site, but the right target site duplication is (GT)AGGG (Fig. 6). Three of the four short inverted repeats flanking the tandem inside the target site duplications and flanking ORF213 on one side of the central TAAGGG are TGAA, while TTAA lies on the other side. The same sequence deviations were also found in the chromosomal copy of the tandem, indicating that the plasmid copy derives from the chromosome (see below).

A third large CP, pING6 (Fig. 1 and 2), differs from pING4 in that it contains a second copy of the tandem which is in-

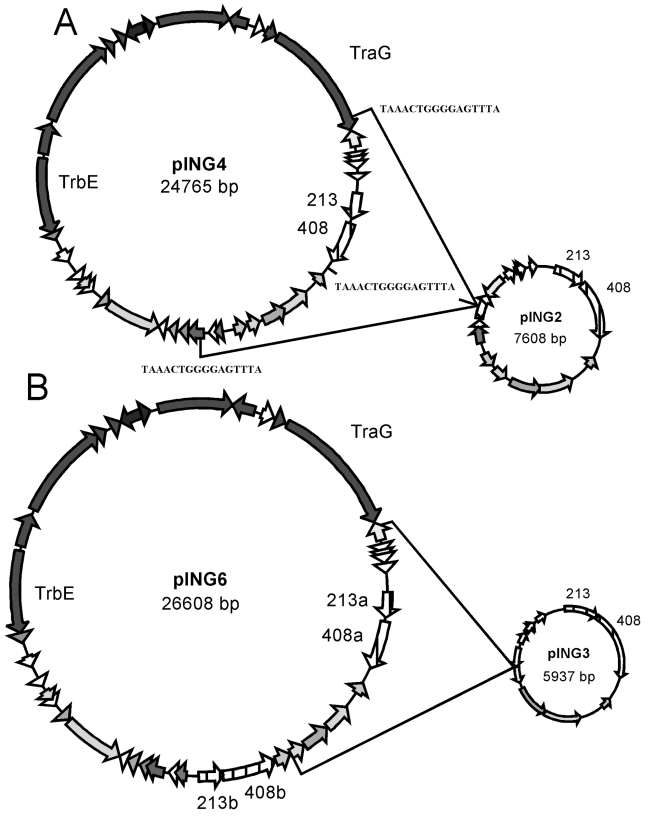

FIG. 4. ORF maps showing, schematically, the generation of the small CP pING2 from the large CP pING4 (A) and pING3 from the large CP pING6 (B). The sequences probably responsible for the generation of pING2 are shown in panel A.

TABLE 1. ORFs in pING1 and their homologs in pNOB8

pING1 ORF	pNOB8 ORF	% Identity	Assignment
153a	165	82.2	
111			
99	87	87.2	
1042	1025	77.5	TraG
153b	142	17	
69[a]			
65a[a]			
65b[a]			
64[a]			
55[a]			
57			
320			
56[a]	50b	33	(*mob*)
218[a]	246	25.9	(*mob*)
198[a]	253	43.7	(*mob*)
95[a]	94	29.2	(*mob*)
97	94	31.8	
106a	108	63.2	
54			
165	164	75	
61	72b	57.4	
92	92	47.8	
94			
457	439	27.3	
73	80	47.9	
51			
88	87	22.1	
68			
98			
53	249	52.8	
640	630b	89.2	TrbE
298	312	80.6	
779	778	81.4	
86	86	83.7	
106b	109	85.8	
147	147	90.4	
52	52b	92.3	
622	604	85.2	

[a] ORF in pING3 sequence.

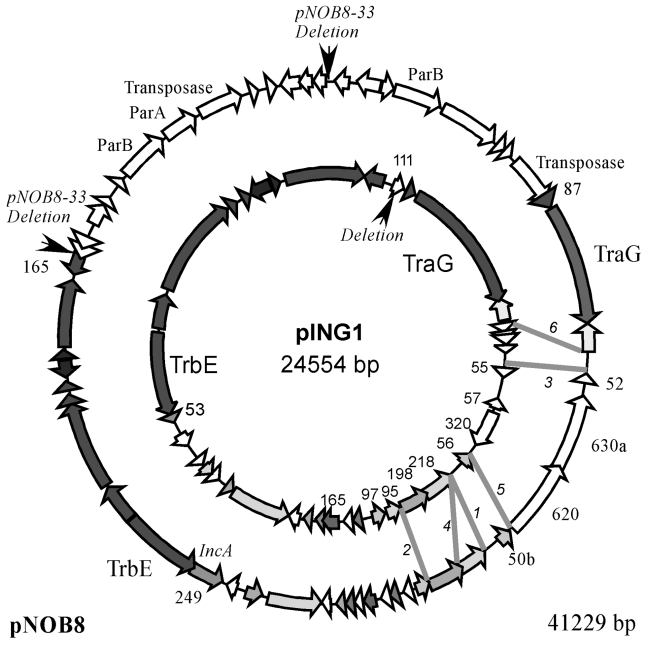

FIG. 5. Comparative ORF maps of the *Sulfolobus* CPs pING1 (inner circle) and pNOB8 (outer circle [13]). ORFs discussed in the text are labeled; homologous ORFs are shaded in proportion to their sequence identity (calculated from the pING1 sequence). Highly similar DNA sequence motifs in the region of the pING2 and pING3 plasmids are marked as lines between the sequences and numbered, in italics, as in Table 2. The putative deletion in pING1 is also noted. The maps are not to scale.

serted, without replacement, at a target site TGGGGG which is conserved at the left flank, with a central sequence TAA GGG and a right flanking sequence GTAGGG, as occurs in both pING4 and the chromosome (Fig. 6). The deviations of the target site duplications and inverted repeats from the ideal sequences were the same as for the first tandem copy. Thus, this second tandem appears to be derived either from the first copy or from the host chromosome. A sequence ACAAACG immediately downstream of the right target site duplication also occurs in both tandem copies (Fig. 6) and in the chromosome.

Small, functionally defective plasmids. The defective small plasmids pING2 and pING3 (Fig. 3 and 4) are identical in sequence to regions in the large plasmids pING4 and pING6, respectively. Both contain five colinear ORFs which are similar to colinear ORFs in the pNOB8 genome (Fig. 5). Noncoding stretches occur between ORF218 and ORF50b, downstream from ORF50b, and upstream of the tandem which lies between the fourth and fifth ORFs (Fig. 2 and 5). Therefore, we conclude that neither ORF55, ORF57, and ORF320 in pING1, which were replaced by the tandem in pING4, nor the nonhomologous ORFs located at the same position in pNOB8 are required for conjugation.

Since pING2 and pING3 are unable to effect their own conjugative transfer but can be transferred with the help of complete CPs (see below), their genomes should contain functional motifs, including a putative origin of transfer, *oriT* (6) and, possibly, genes required for mobility (6), which is a precondition for transfer. Five DNA sequence regions occurring mainly between ORFs (Table 2 and Fig. 5), which are highly conserved in the pINGs and pNOB8, are candidates for such functional motifs. Moreover, some of the five shared ORFs in this region may encode additional mobility functions. For the pINGs, the first of these ORFs, ORF95, shows significant sequence similarity to ORF97 immediately upstream, whereas in pNOB8 a small ORF in this location has the opposite orientation and shows no homology to its neighbor.

The small defective pING2 is derived from pING4 (see below) potentially by looping out of the sequence between two copies of a 16-bp repeat (Fig. 4), one in ORF165 and the other in the region encoding the C terminus of TraG. A third copy of this repeat is present in all pINGs between ORF218 and ORF56 and an inverted copy occurs about 100 bp downstream. We have not observed any small, spreadable plasmid that loops

TABLE 2. DNA sequence motifs present in the small plasmids pING2 and pING3 and highly similar sequences in pNOB8

Motif	Positions		Length (bp)	% Identity	Gaps
	pING1	pNOB8			
1	9246–9351	16127–16321	106	83	1
2	10634–10707	17854–17927	74	85	0
3	6425–6456	11002–11033	32	96	0
4	9266–9289	17082–17105	24	100	0
5	8758–8781	15422–15445	24	95	0
6	5694–5767	10455–10527	74	78	1

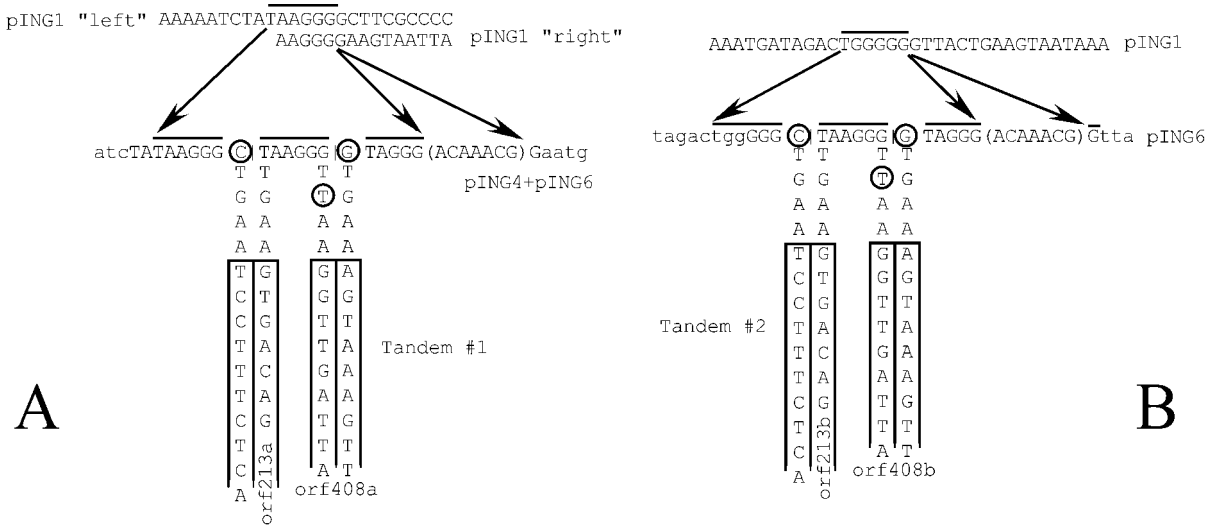

FIG. 6. Sites of integration of the tandem arrays of IS elements, containing ORF213a/b and ORF408a/b, from the *S. solfataricus* P1 chromosome in pING1 and the sequences in and around these sites in the products of integration, pING4 (A) and pING6 (A and B), respectively. The sequences in the products which are not present in the *S. solfataricus* genome (She and Garrett, unpublished) are in lowercase. The target sites for both insertion events are overlined. Deviations in sequence between the short inverted repeats are circled.

out between either of the flanking copies and the internal copy of the repeat. Moreover, no rational explanation was found for the looping out of pING3 from pING6 (see below).

The variation of pING1 in vivo by transpositions of an IS element and by the looping out of small defective plasmids. The plasmids produced in several consecutive steps of 50-fold growth (see Materials and Methods) of *S. solfataricus* P1 conjugated with pING1 were characterized by their *Eco*RI restriction patterns. pING1 was completely replaced by pING4 and the small defective pING2 after the second growth passage (Fig. 3, lanes 3 to 5). A comparison of the intensities of the *Eco*RI restriction fragments of pING2 (arrows between lanes 9 and 10) and pING1+4 (common band between the two lower arrows) shows that after the second passage there are about five copies of pING2 per copy of pING4. The complete disappearance of pING1 is demonstrated by the absence of the third band from the top of its digestion pattern (lane 1) in lane 4. When the growth passages were started with pING4, the looping out of pING2 led to an even greater relative abundance of pING2 (lanes 7 to 9). After the third growth passage, pING2 was always the predominant plasmid.

Restriction fragments excised from two internal *Eco*RI sites in the looped-out region of pING2 and pING3 were the same, but those created by joining of the flanking sequences after looping out were different (Fig. 3, arrows). In addition to these major characteristic bands, we observed several minor fragments indicating the formation of additional spreadable plasmids by other looping out processes.

pING3 requires pING6 for transfer. The end product after two growth passages of pING6 was almost pure pING3 (Fig. 3, lane 11). However, after a third passage, the culture appeared to be cured of the small plasmid, establishing that the helper was required for spreading. Moreover, the cured cells were temporarily resistant to conjugation by the large pINGs (8). In the fourth passage, pING3 and pING6 reappeared, apparently because they could spread from a small residual population of transcipients after the loss of the temporary immunity of the cured cells (not shown).

DISCUSSION

Elucidating the mechanism of conjugation in *Sulfolobus* has been hindered by a lack of available genetic approaches and the absence of methods which allow dissection of the individual steps in the process. Therefore, we have tried to assess the role of ORFs in *Sulfolobus* CPs in conjugation by comparing DNA sequences of two different subfamilies of CPs (8): the pNOB8 subfamily, comprising the low-copy-number plasmid pNOB8 and its high-copy-number derivative pNOB8-33 (13), and the pING subfamily, containing functional and defective variants of a high-copy-number CP (8).

Plasmid pNOB8 is stably maintained and correctly partitioned in its natural host NOB8H1 but not in the foreign host *S. solfataricus* P1, which implicates both the host and plasmid genomes in these processes. The functional pING variants pING1, pING4, and pING6 lack homologs of 21 contiguous ORFs of pNOB8 including ParA and two ParB homologs (Fig. 5). Since all of these plasmids can undergo conjugative transfer, apparently none of these ORFs are essential for the transfer process, which includes pair formation between donor and recipient and transfer and mobility functions. However, some of these ORFs appear to be required for copy number control; the ParA and ParB homologs probably participate in partitioning and, therefore, maintenance. In addition to the two transposases in pNOB8, which have probably been transposed from the *Sulfolobus* chromosome (13), we have observed the highly efficient substitutive transposition of a tandem array of chromosomal IS elements into pING1 to yield pING4. The resulting deletion of three ORFs from pING1 had no detectable effect on the functionality of the plasmid. These ORFs are therefore also not essential for conjugative transfer. Thus, the pINGs appear to be defective plasmids which have retained genes required for conjugative spreading but have lost functions, especially controls, which involve interaction with their hosts.

The striking conservation of the almost contiguous cluster of ORFs flanked by ORF53 and the TraG homolog in the pING plasmids suggests that these are the essential transfer genes.

The defective plasmids pING2 and pING3, derived from the large functional plasmids pING4 and pING6, respectively, lack this ORF cluster and cannot transfer themselves but are efficiently transferred with the help of their mother plasmids. Their preferential transfer and replication may reflect their smaller sizes.

The core regions of pING2 and pING3 yield the same two *Eco*RI restriction fragments as the mother plasmids pING4 and pING6, respectively, and are thus considered to be identical (Fig. 3). The third *Eco*RI fragment results from joining of the ends of the flanking sequences which loop out from the mother plasmids. Its larger size in pING2 reflects the longer flanking sequences. Further weaker bands probably arise from the formation of additional defective plasmids. For pING4 the flanking sequences end in two copies of a 16-bp repeat which provide recombination sites for looping out of pING2, but we identified no such sequence elements which would account for the efficient formation of pING3.

The flanking copies of the repeat probably involved in the formation of pING2 are also present in both pING1 and pING6, but corresponding small plasmids were not observed from either of them. As pING4 is very efficiently formed from pING1 by transposition of the tandem IS elements (Fig. 3), this transposition presumably also occurs in the smaller plasmid produced from the repeats in pING1, so that only pING2 is observed as a stable end product. No plasmid was derived from the two flanking repeats in pING6, possibly due to recombination between the two tandem IS elements which would be present on the small plasmid, leading to the deletion or disruption of essential ORFs or motifs (see below).

A third copy of the repeated sequence is present in the central part of the region forming pING2 (Fig. 4). Although pING2 is produced very efficiently, there is no evidence for the formation of smaller plasmids by looping out between a terminal and the central copy of the repeat. This indicates that the minimal complement of sites and functions required for producing defective but transferable plasmids includes DNA sequence on both sides of the central copy of the repeat, e.g., the putative replication origin for transfer (*oriT* in bacterial conjugative systems) and/or genes essential for accessory mobility functions. Only four of the five ORFs in this region show significant protein sequence similarity to ORFs in the corresponding region in pNOB8. This similarity ranges between 26 and 44% and is thus clearly lower than that between corresponding ORFs in the highly conserved region presumed to encode transfer functions (see above). In contrast, six motifs in this region mostly not located in ORFs (Fig. 5) show DNA sequence identities as high as 90%. They are candidates for elements of an *oriT*. Altogether these findings support the view that conjugation in archaea, as in bacteria, involves DNA replication as was already suggested by the selectivity and unidirectionality of the transfer (8, 10). The defective spreadable plasmids which are efficiently generated, preferentially transferred, and thereafter replicated to high copy number provide a means of further dissecting the mechanism of this process.

The recurrent spreading of pING3 after it appeared to have been lost by curing shows that the immunity toward superconjugational transfer of an incompatible CP is also conferred by the defective plasmids. This effect is lost on further growth after curing. As shown previously, immunity of transcipients to superconjugation is restricted to plasmids belonging to the same subfamily (8). The pINGs and the pNOBs constitute different subfamilies and are thus compatible with each other. Comparison of the ORFs in the spreadable defective plasmids with those in the corresponding region of pNOB8 could thus help to identify the genes conferring immunity. If, as suggested, the highly conserved noncoding DNA sequences in the compatible pINGs and pNOBs constitute *oriT*s and/or other sequence motifs involved in targeting transfer, then the factor(s) conferring immunity is likely to act on cell-cell interaction rather than on transfer. This could be estimated quantitatively by studying pair formation between cells containing compatible or noncompatible plasmids. It has indeed been shown previously that pair formation between donor cells harboring pNOB8 is almost as infrequent as that between empty recipient cells (10).

The deficient but spreadable CPs, although initially propagated to high copy number after transfer, are not maintained. Such a total block in replication after multiplying to high copy number, which allows curing, has also been observed for the large pINGs and other defective CPs (e.g., pSOG2/4B [I. Holz and W. Zillig, unpublished data]. The cause is unknown. This lack of maintenance limits the experimental use of these plasmids for vector development. However, it should be possible to construct maintainable spreadable plasmids by adding maintenance functions from other plasmids and to use them as vehicles to transport DNA sequences for homologous recombination, e.g., for gene disruption experiments.

The efficient in vivo generation of pING4 from pING1 by transposition of an element from the chromosome, and the efficient in vivo generation of the defective but spreadable plasmids pING2 and pING3 from pING4 and pING6, respectively, show that transpositions and other site-specific recombinations occur reproducibly and frequently both from the *Sulfolobus* genome and between plasmids. This can readily be observed when the change does not lead to plasmid loss by inhibition of function and, especially, when a selective advantage is conferred. For example, when CPs were spread by conjugative transfer, the observed variation was small because any genetic change causing loss of an essential transfer function escaped observation. However, when propagation was achieved by growth of transcipients, the variety of recombinants observed was large. This process appeared to be further stimulated by stress.

The recently sequenced genome of *S. solfataricus* P2 contains a number of different IS elements, many of which are likely to be mobile (5, 12). The tandem repeats that insert into the pING plasmids, identified here, derive from the closely related chromosome of *S. solfataricus* P1. It has been shown that at least some of these IS elements induce genetic variation (11). Moreover, the strains vary greatly in sequence, copy number, and distribution (which has even been shown to vary geographically [7]). IS elements contribute greatly to genome plasticity, which appears to be widespread in nature (4) but is not well understood. Characterization of the variation of the pING plasmids due to the presence IS elements allows investigation of genomic variation due to IS elements in a relatively simple system.

Conclusions. This characterization and analysis of the pING family of conjugative plasmids from *Sulfolobus* has provided insights into which proteins and DNA sequence motifs are involved in conjugation in archaea. Comparison with the pNOB8 plasmid has allowed the preliminary assessment of the importance of different ORFs for conjugation. The discovery and analysis of small functionally defective plasmids has led to the localization of the ORFs and sequence motifs probably involved in plasmid mobilization.

ACKNOWLEDGMENTS

This investigation was supported by the European Union in the framework of its Biotechnology Program project "Extremophiles as Cell Factories" and by the Novo Nordisk Foundation. K.M.S. was

supported by a Marie Curie research fellowship from the European Union and a NSF-NATO postdoctoral fellowship.

We thank our former colleague H. P. Arnold for valuable help in analyzing the data.

REFERENCES

1. **Altschul, S., T. Madden, A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Arnold, H. P., Q. She, H. Phan, K. Stedman, D. Prangishvili, I. Holz, J. K. Kristjanson, R. A. Garrett, and W. Zillig.** 1999. The genetic element pSSVx of the extremely thermophilic crenarchaeon *Sulfolobus* is a hybrid between a plasmid and a virus. Mol. Microbiol. **34:**217–226.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
4. **Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277:**1453–1474.
5. **Charlebois, R., Q. She, D. Sprott, C. Sensen, and R. Garrett.** 1998. *Sulfolobus* genome: from genomics to biology. Curr. Opin. Microbiol. **1:**584–588.
6. **Lanka, E., and B. Wilkins.** 1995. DNA processing reactions in bacterial conjugation. Annu. Rev. Biochem. **64:**141–169.
7. **Martusewitsch, E., C. W. Sensen, and C. Schleper.** 2000. High spontaneous mutation rate in the hyperthermophilic archaeon *Sulfolobus solfataricus* is mediated by transposable elements. J. Bacteriol. **182:**2574–2581.
8. **Prangishvili, D., S. V. Albers, I. Holz, H. P. Arnold, K. Stedman, T. Klein, H. Singh, J. Hiort, A. Schweier, J. K. Kristjansson, and W. Zillig.** 1998. Conjugation in Archaea—frequent occurrence of conjugative plasmids in *Sulfolobus*. Plasmid **40:**190–202.
9. **Prangishvili, D., I. Holz, E. Stieger, S. Nickell, J. K. Kristjansson, and W. Zillig.** 2000. Sulfolobicins, specific proteinaceous toxins produced by strains of the extremely thermophilic archaeal genus *Sulfolobus*. J. Bacteriol. **182:**2985–2588.
10. **Schleper, C., T. Holz, D. Janekovic, J. Murphy, and W. Zillig.** 1995. A multicopy plasmid of the extremely thermophilic archaeon *Sulfolobus* effects its transfer to recipients by mating. J. Bacteriol. **177:**4417–4426.
11. **Schleper, C., R. Roeder, T. Singer, and W. Zillig.** 1997. An insertion element of the extremely thermophilic archaeon *Sulfolobus solfataricus* transposes into the endogenous beta-galactosidase gene. Mol. Gen. Genet. **243:**91–96.
12. **She, Q., F. Confalonierei, Y. Zivanovic, N. Medina, A. Billault, M. Awayez, H. Phan Thi-Ngoc, B. Thi Pham, J. Van der Oost, M. Duguet, and R. Garrett.** 2000. A BAC library and paired-PCR approach to finishing the genome sequence of *Sulfolobus solfataricus* P2. DNA Sequence **11:**183–192.
13. **She, Q. X., H. E. Phan, R. A. Garrett, S. V. Albers, K. M. Stedman, and W. Zillig.** 1998. Genetic profile of pNOB8 from *Sulfolobus*—the first conjugative plasmid from an archaeon. Extremophiles **2:**417–425.
14. **Zillig, W., H. P. Arnold, I. Holz, D. Prangishvili, A. Schweier, K. Stedman, Q. She, H. Phan, R. Garrett, and J. K. Kristjansson.** 1998. Genetic elements in the extremely thermophilic archaeon *Sulfolobus*. Extremophiles **2:**131–140.
15. **Zillig, W., A. Kletzin, C. Schleper, I. Holz, D. Janekovic, J. Hain, M. Lanzendoerfer, and J. K. Kristjansson.** 1994. Screening for Sulfolobales, their plasmids and their viruses in Icelandic solfataras. Syst. Appl. Microbiol. **16:**609–628.