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A Multicopy Plasmid of the Extremely Thermophilic Archaeon *Sulfolobus* Effects Its Transfer to Recipients by Mating

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A plasmid of 45 kb, designated pNOB8, was found in high copy number in a new heterotrophic *Sulfolobus* isolate, NOB8H2, from Japan. Dissemination of the plasmid occurred in six cultures of nine different *Sulfolobus* strains when small amounts of the donor were added. These mixed cultures exhibited a high average copy number of the plasmid, between 20 and 40 per chromosome, and showed a marked growth retardation. Horizontal transfer of pNOB8 was proved by isolating transcipients from mating mixtures via single colonies. In these isolates, the copy number of the plasmid appeared to be subject to a control mechanism. Cell-free filtrates of donor cultures did not transmit the plasmid, and plating of the donor on lawns of recipients did not result in plaque formation, suggesting that the transfer was not mediated by a virus. Rapid formation of cell-to-cell contacts between differently stained donor and recipient partners was demonstrated after the two strains were mixed. Electron microscopic analysis of mating mixtures revealed many cell aggregates made up of 2 to 30 cells and intercellular cytoplasmic bridges connecting two or more cells. Cells that had been transformed with purified plasmid DNA as well as transcipients isolated from mating mixtures were shown to serve as donors for further transmission of pNOB8. The plasmid undergoes extensive genetic variations, since deletions and insertions were frequently observed in plasmid preparations from the donor strain and from mating mixtures.

In contrast to other processes of horizontal gene transfer, such as phage transduction and natural transformation, conjugation effects gene transfer between bacteria via specific, physical contacts between donor and recipient cells. There is an increasing appreciation for the role of conjugation in the horizontal spread of genes (e.g., antibiotic resistance genes [4]) and in the evolution of genomes, as it has been demonstrated to occur even between widely divergent bacterial species (1, 11, 21) and, more recently, also between bacteria and eucaryotes (8, 19). DNA transfer is usually mediated by large conjugative plasmids that are mobilized at a high frequency and that may also cotransfer chromosomal genes or smaller, nonconjugative plasmids (for reviews, see references 3, 22, and 23). Conjugative plasmids have been detected in a variety of gram-negative and gram-positive bacteria. Different types encode quite dissimilar mating systems, diverging, for example, in breadth of host range and in the mechanism of cell-to-cell contact formation between donor and recipient. However, there is increasing evidence that the mechanism of genetic transfer from even very divergent conjugative plasmids is conserved (reference 6 and references therein), including Ti plasmid-mediated DNA transfer to plant cells (10).

Our knowledge about horizontal gene transmission in the domain *Archaea* (24) (formerly *Archaebacteria*) is very limited. A transducing phage has been detected in a methanogen (12), and Mevarech and Werczberger (13) have described a mating system in the archaeon *Haloferax volcanii*: cells growing on solid surfaces exchange genetic material, which results in a recombinant chromosomal genotype of the conjugation progeny as scored by using different auxotrophic mutants as parental types. This system differs from classical bacterial conjugation in that it is bidirectional and seems to work via intercellular cytoplasmic bridges (15). Pairs of cells connected by one or more bridges and even networks of cells connected by intercellular bridges in mating mixtures have been directly documented by scanning electron microscopy. Recombinant selectable plasmids were also shown to be mobilized between *H. volcanii* cells as well as from *H. volcanii* to the related *Haloferax mediterranei* (20).

Here, we present the first description of an archaeal plasmid that can be transmitted horizontally. It propagates efficiently in liquid cultures of strains of the extreme thermophile *Sulfolobus* via a cell-to-cell contact-dependent transfer mechanism. The plasmid is quite unusual in that it can replicate up to very high copy numbers in its original host strain and in mating mixtures, thereby greatly affecting cell growth. In addition, we show that when propagated under laboratory conditions, this plasmid appears to undergo extensive genetic variation.

MATERIALS AND METHODS

Sampling and isolation of *Sulfolobus* strains. Samples were taken from different hot springs and mud holes at Hakone and in Hokkaido, Japan, as described by Zillig et al. (25). The isolation of heterotrophic *Sulfolobus* strains from enrichment cultures was also done as described previously (25).

***Sulfolobus* strains and culture conditions.** All *Sulfolobus* strains used are listed in Table 1. The salt base of the medium was made either by the method of Brock et al. (2) or by the method of Zillig et al. (25). The medium was supplemented with 0.2% tryptone as a carbon source and adjusted to pH 3 with sulfuric acid. Fifty-milliliter liquid cultures were incubated in long-necked Erlenmeyer flasks at 80°C under moderate shaking. Plating of single colonies on Gelrite (Kelco, San Diego, Calif.) plates and the plaque assay using a soft layer have been described previously (17). The plates were incubated at 80°C, and colonies were scored after 5 to 10 days.

DNA analysis. The preparation of DNA from *Sulfolobus* cells has been described previously (25). After cell lysis and phenolization, the DNA was first banded in a CsCl gradient in the presence of small amounts of ethidium bromide (5 μg/ml), yielding total DNA, i.e., chromosomal and plasmid DNAs. A second

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TABLE 1. Strains used

Strain(s)[a]	Origin	Reference	Propagation of pNOB8
Sulfolobus sp. strains NOB8H2 and NOB8H3[b]	Noboribetsu, Japan	This study	None (host)
S. solfataricus P1, DSM 1616	Pisciarelli, Italy	26	+
S. solfataricus (SSV1)		17	+
S. solfataricus PH1[c]		18	+
S. islandicus KVEM10H1[d]	Kverkfjöll, Iceland	25	+
S. islandicus REN1H1(pRN1, pRN2)	Reykjanes, Iceland	25	+
S. islandicus HEN7H2(pHE7)	Hengill, Iceland	25	+
Sulfolobus sp. strain DAI2H2[e]	Daisetzusan, Japan	This study	+
S. shibatae (SSV1) DSM 5389	Beppu, Japan	7	−
S. acidocaldarius DSM 639	Yellowstone National Park	2	−
Sulfolobus sp. strain 59/2[f]	Ketatahi, New Zealand	Unpublished	−

[a] All strains are heterotrophic isolates of *Sulfolobus*. Plasmids or viruses harbored by the strains are indicated in parentheses.
[b] Two new isolates from Japan in which plasmid pNOB8 has been detected (Fig. 1).
[c] β-Galactosidase mutant of *S. solfataricus* P1.
[d] Host for virus SIRV (25).
[e] New isolate from Japan, clearly distinct from NOB8H2 and NOB8H3 as judged by comparison of DNA restriction fragment patterns.
[f] New isolate harboring plasmid p59/2.

gradient using 1 mg of ethidium bromide per ml was used to separate covalently closed circular DNA (cccDNA) from chromosomal DNA plus open circular and linearized plasmid forms. For electrophoretic analysis, 2 μg of chromosomal or total DNA or 1 μg of cccDNA was cut with the appropriate restriction enzyme (Boehringer Mannheim) and separated on a 1% agarose gel (16). Southern hybridizations with [α-^{32}P]ATP-labelled DNA probes (5) were performed by standard procedures (16). The filters were washed under stringent conditions. The copy number of the pNOB8 plasmid was determined by loading total DNA onto a 0.7% agarose gel in order to separate the chromosomal DNA from the main fraction (open circular form) of the plasmid. After the gel was stained with ethidium bromide, the DNA bands were quantitated. For calculation, the *Sulfolobus* chromosome was taken as 2.7 Mb (9). Since the fraction of sheared plasmid DNA was not separated from chromosomal DNA, the approximate copy number of pNOB8 determined by this method is an underestimate.

To test the stability of free DNA under the growth conditions of *Sulfolobus* cells, 5 μg of total DNA of strain NOB8H2 was incubated at 80°C in 100 μl of growth medium and in water as a control. After 1, 2, and 5 h, samples were removed, phenolized, precipitated, and analyzed on a 1% agarose gel.

Mating procedure. Mating mixtures were set up from logarithmically growing cultures of donor and recipient strains (usually 10^8 cells per ml) whose density was exactly determined by cell counting of aliquots in the light microscope prior to the making of the mixture. Mixtures (donor/recipient cell ratio, 1:1,000 or 1:1) were made in fresh medium to give a cell concentration of 10^7/ml and were subsequently incubated under moderate shaking, as is standard. Samples were removed at the times indicated below and were either plated or used for preparations of total DNA and for determination of the optical density.

To investigate the possibility of phage-mediated plasmid transfer, cell-free filtrates of donor cultures were prepared by low-speed centrifugation of a growing culture and subsequent filtration of the supernatant through a membrane filter (0.45-μm pore size; Millipore). Samples (1 and 3 ml) of the filtrate were mixed with recipient cells (10^7/ml) and treated as described for mating mixtures.

Staining of *Sulfolobus* cells with fluorescent dyes. Logarithmically growing cells of donor and recipient strains were concentrated by centrifugation and subsequent resuspension in growth medium to a density of 2×10^8 cells per ml. Fluorescent dye (Chromomycin A3 or Hoechst no. 33258; Sigma) was added to a final concentration of 5 μg/ml, and the cells were incubated for 45 min at 80°C. After two washes with growth medium, they were resuspended in fresh medium in the original volume and mixed in a 1:1 ratio (1 or 2 ml, final volume). The mating mixture was incubated at 80°C, and samples were taken at different times. For microscopic analysis, 20-μl aliquots were mixed with 10 μl of hot, dissolved agarose and poured onto the mount. The staining was visualized by fluorescence microscopy in a Zeiss axiophot microscope.

Electron microscopy. Cells were visualized in a Zeiss 109 electron microscope after negative staining with 1 to 2% uranyl acetate.

Transformation of *Sulfolobus* cells by electroporation. A 200- or 300-ng sample of plasmid DNA was used for transformation of *S. solfataricus* P1 as described elsewhere (17). After electroporation, the cells were grown in liquid culture, and samples were removed for plating or for preparation of total DNA at the times indicated below.

RESULTS

Isolation of plasmid pNOB8. Samples taken from acidic hot springs and mud holes of different solfataric fields at Hakone and in Hokkaido, both in Japan, were enriched in liquid culture and plated. A total of 46 *Sulfolobus* strains were isolated from single colonies, representing about 14 different types distinguished from each other and from known *Sulfolobus* species by restriction fragment patterns of their chromosomal DNAs (not shown). The total DNAs of two of these isolates (NOB8 H2 and NOB8H3), belonging to the same type and originating from the same source, each yielded a set of additional restriction fragments in high copy number compared with the fragments derived from the chromosome (Fig. 1; lanes a show *Eco*RI cuts of total DNAs). These fragments could be assigned to an extrachromosomal genetic element (pNOB8) that was isolated as cccDNA by rebanding the total DNAs in CsCl gradients in the presence of 1 mg of ethidium bromide per ml. Figure 1, lanes c, shows *Eco*RI cuts of the purified plasmid DNAs; lanes b show the corresponding cuts of the DNAs of lower density from the same gradient which contained the chromosomal DNAs plus the open circular and the linear forms of the plasmids. Restriction of the plasmid DNA from strain NOB8H2 yielded a regular pattern of fragments in an equimolar ratio (H2 lane e). The sum of the sizes of these fragments was about 45 kb. The pattern for NOB8H3 was slightly different, showing an additional, substoichiometric fragment, apparently at the expense of a smaller fragment by an insertion of about 1.5 kb (Fig. 1, H3 lane e, arrows). Southern analysis proved that these two fragments do indeed share homologous sequences (not shown). The copy number of pNOB8 in these cultures was determined to be around 20 per chromosome. The generation time of the host that was used for all following experiments, NOB8H2, was 16 h. This is at least twice as long as the generation times of other known *Sulfolobus* strains (between 4 and 8 h). Because of its size and high copy number, we considered the possibility that pNOB8 might represent the DNA of a virus. However, careful inspection of the culture in the electron microscope did not reveal any extracellular particles resembling virus (or phage) particles. Even specific enrichment for putative particles, i.e., polyethylene glycol precipitation and CsCl gradient centrifugation, did not yield virus-like structures.

Dissemination of pNOB8 in cultures of different *Sulfolobus* strains. Attempts to infect various *Sulfolobus* strains with filtered culture supernatants of NOB8H2 failed completely, as expected from our inability to visualize virus-like particles. A control, with small amounts of NOB8H2 cells added, however,

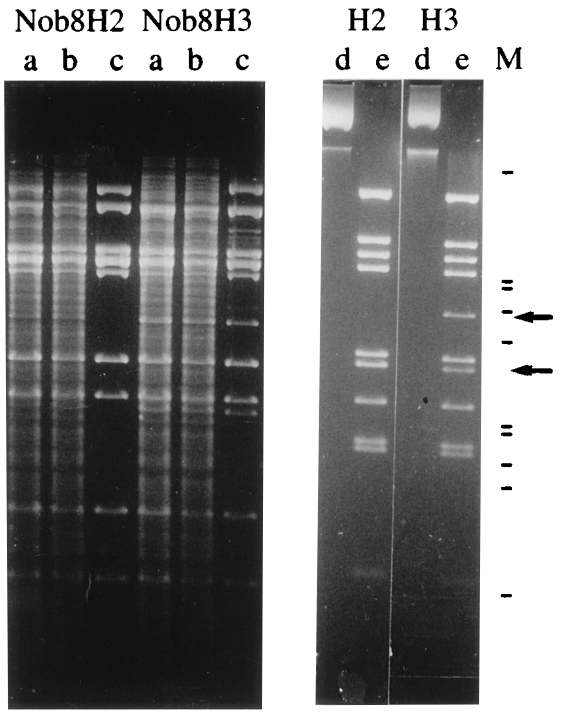

FIG. 1. DNAs isolated from NOB8H2 (H2) and NOB8H3 (H3). Lanes a, total DNA; lanes b, upper band of second CsCl gradient containing chromosomal DNA plus open circular and linearized DNAs of the plasmid; lanes c to e, lower band of the same gradient containing cccDNA of plasmid pNOB8. DNAs were cut with *Eco*RI (lanes a to c), uncut (lanes d), or cut with *Bam*HI (lanes e). Lane M, size marker (λ cut with *Eco*RI and *Hin*dIII). An additional, substoichiometric fragment and a smaller fragment at whose expense it apparently was created are indicated (H3 lane e, arrows).

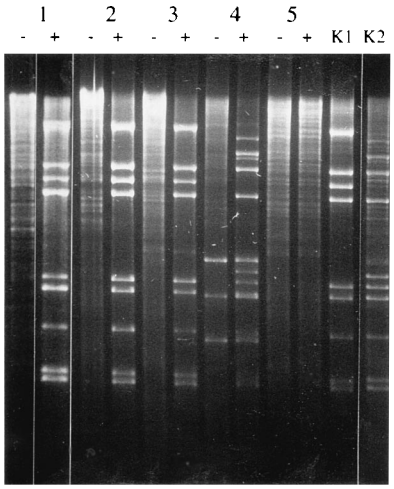

FIG. 2. Total DNAs from cultures of different *Sulfolobus* strains that have (+) or have not (−) been mixed with donor strain NOB8H2 in a donor/recipient cell ratio of 1:1,000. The DNAs were prepared after 2 days, and 2 μg of each was cut with *Bam*HI. Lanes: 1, *S. solfataricus* P1; 2, *S. islandicus* KVEM10H1; 3, *Sulfolobus* sp. strain DAI2H2; 4, *S. islandicus* REN1H1, with plasmids pRN1 and pRN2 visible as three overstoichiometric fragments; 5, *S. shibatae*; K1 and K2, preparations of total DNAs from different cultures of donor strain NOB8H2.

led to strong propagation of the plasmid in cultures of many *Sulfolobus* strains. In the experiments whose results are shown in Fig. 2, one cell of NOB8H2 was added per 1,000 recipient cells. Total DNAs of the cultures as well as of controls from the donor and recipients were prepared after 2 days of growth. In six of nine of such mixed cultures, we observed strong production of plasmid pNOB8 (Table 1 and Fig. 2, lanes 1+ through 4+) and a marked growth retardation compared with the growth of the controls (not shown). In the three cases in which no propagation of the plasmid was observed (Table 1 and Fig. 2, lane 5+, for *S. shibatae*), growth of the strains was unaffected (not shown). In all positive cases, the plasmid DNA was found in large amounts, with an average copy number as high as or sometimes even exceeding that of the original donor strain (between 20 and 40 per chromosome). In some cases, however, variants of the plasmid appeared in the donor strain that was grown as a control in parallel (Fig. 2, lane K2) as well as in the corresponding mating mixtures (Fig. 2, lane 4+; see "Variants of pNOB8" below).

We regarded these results as a strong suggestion that the plasmid is transmitted to different *Sulfolobus* strains in a cell-to-cell contact-dependent mechanism. The possibility that the donor strain simply overgrew the recipient seemed unlikely because of the low growth rate of the former and could be excluded in those cases in which other indigenous extrachromosomal elements present in the recipient were clearly visible in the DNA preparation of the mixed culture. This can be seen in the mixed culture with strain REN1H1 (Fig. 2, lanes 4− and 4+), which harbors plasmids pRN1 and pRN2, seen as three *Bam*HI fragments occurring in much higher copy number than chromosomal bands.

To further check the possibility of involvement of a virus in the transmission of the plasmid DNA, the mixed cultures of donor and recipients in which strong propagation of pNOB8 had been observed were inspected in the electron microscope, but again no virus particles could be identified. Furthermore, we observed no plaque formation when cells or culture supernatants of NOB8H2 were plated on lawns of different recipient strains.

In order to find out whether transmission of the plasmid by natural transformation is possible, we examined the stability of free DNA under the growth conditions of *Sulfolobus* cells, i.e., 80°C and a growth medium pH of 3. DNA incubated under these conditions was found to be completely degraded within 1 h. Furthermore, the mere addition of purified pNOB8 DNA to growing cultures of a recipient did not result in spread of the plasmid.

Isolation of transcipients. In order to prove transfer of pNOB8 into other *Sulfolobus* strains, we isolated transcipients (recipients containing transmitted plasmid) by plating mixed cultures of *S. solfataricus* × NOB8H2 and of *S. islandicus* HEN7H2 (harboring plasmid pHE7) × NOB8H2. Whereas the plating efficiency of either recipient alone was almost 100% and the colonies were large (approximately 2 to 5 mm in diameter), as usual, plating of the donor strain NOB8H2 alone was inefficient (<10%), yielding much smaller colonies. Even less efficient was the plating of mating mixtures 2 days after growth in liquid culture, yielding only tiny, sometimes hardly visible colonies (<1 mm). We picked single colonies from the mating mixtures under a binocular microscope and grew them in liquid culture. As shown in Fig. 3A, *Bam*HI digests of total DNAs from these clones each exhibited the restriction fragment pattern typical for chromosomal DNA of *S. solfataricus* (lanes 4 and 5) and for chromosomal and plasmid DNAs from *S. islandicus* (lanes 1 to 3), respectively, and in addition fragment patterns characteristic for pNOB8 or variants thereof (compare the authentic pNOB8 pattern in control lane K1). Figure 3B shows a Southern hybridization of the same gel

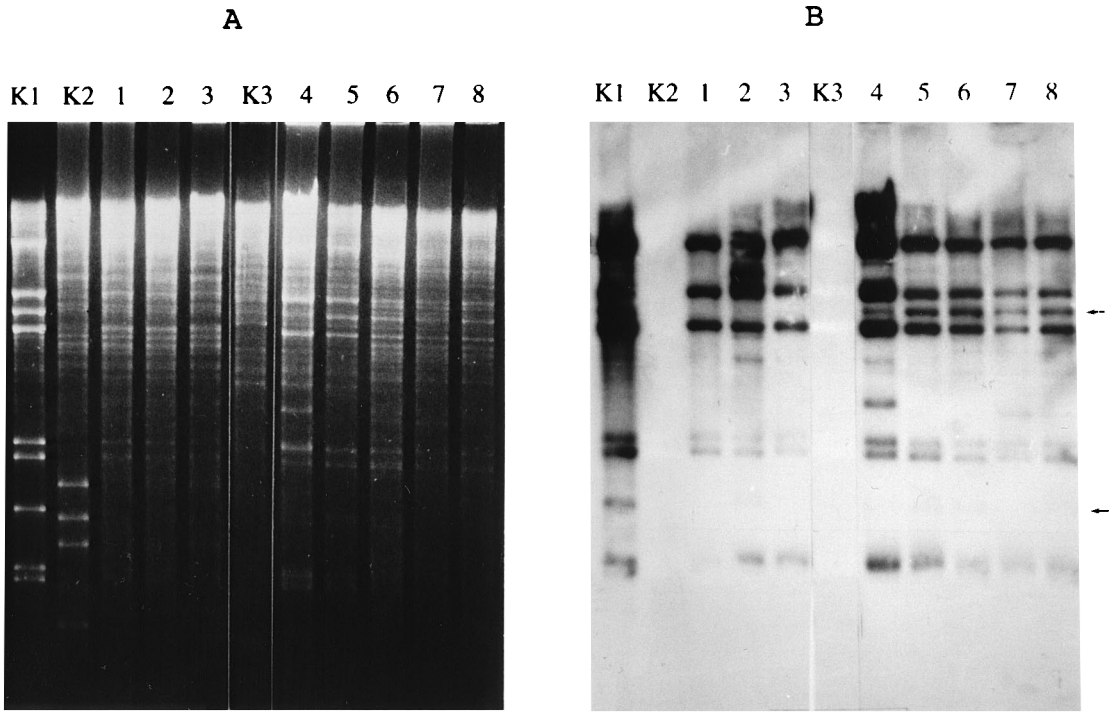

FIG. 3. *Bam*HI digests of total DNAs (A) and corresponding Southern analysis with randomly labelled pNOB8 plasmid used as a probe (B). The DNAs were prepared from independent isolates that were recovered by plating mating mixtures. Lanes: K1, NOB8H2 control; K2, *S. islandicus* HEN7H2 (containing plasmid pHE7, visible as three overstoichiometric fragments) control; 1 to 3, transcipients of *S. islandicus* HEN7H2; K3, *S. solfataricus* PH1 control; 4 and 5, transcipients of *S. solfataricus* PH1; 6 to 8, single isolates of donor strain NOB8H2 isolated by the same procedure. Two *Bam*HI fragments missing in the HEN7H2 transcipients are indicated (panel B, arrows).

using randomly labelled pNOB8 as a probe. In about half of the transcipients analyzed, all of the *Bam*HI fragments of the plasmid were detected (Fig. 3B, lanes 4 and 5, although not all fragments are visible on the exposure shown), whereas in the other cases, the restriction pattern varied from that observed in the host strain, mainly by lacking two *Bam*HI fragments (lanes 1 to 3, arrows). This deletion and other variants of the plasmid were found in several plasmid preparations from cultures of strain NOB8H2 and from mating experiments (see below).

In all the transcipients and also in the isolates of the original donor strain NOB8H2 that were obtained by plating the conjugation mixtures (Fig. 3, lanes 6 to 8), the copy number of pNOB8 was much lower than those observed in mating mixtures and in the cultures of NOB8H2 grown from glycerol conserves, i.e., around 2 to 5 instead of more than 20 per chromosome (compare with plasmid band intensities in Fig. 1 and 2). The copy number of the indigenous plasmid pHE7 in the transcipients of strain HEN7H2 (Fig. 3, lanes K2, showing four fragments of pHE7 in high copy number) was equally reduced (Fig. 3, lanes 1 to 3), though the plasmid was still detectable in Southern analysis (not shown).

In order to determine whether a transcipient containing pNOB8 in low copy number can in turn serve as a donor, we mated the transcipient HEN7H2(pNOB8) with HEN7H2 as the recipient. Figure 4 compares the growth of the mating mixture (donor/recipient cell ratio, 1:1,000) with that of the donor and recipient strains alone, with the growth of the high-copy-number donor, NOB8H2, and with the growth of the mating mixture of this strain with the same recipient (Fig. 4). Whereas the generation time of the low-copy-number donor strain was about the same as that of the recipient, mixing the two strains led to growth retardation similar to that observed in conjugation mixtures containing the original high-copy-number donor, NOB8H2. Accordingly, the plasmid was strongly amplified in the mating mixture (Fig. 5, lane 4), whereas its copy number remained low in the donor strain by itself (Fig. 5, lanes 2 and 3). The average copy number fell again after several passages, though not quite to the low level of the donor (lanes 5 and 6 versus lane 3). These results indicate that the growth retardation of mating mixtures and high-copy-number donors is due to the burden imposed by the high plasmid copy number. Conjugation appears to cause strong amplification, whereas growth on a solid support seems to select for a low-copy-number state, as found in the transcipients isolated from mating mixtures.

Kinetics of the mating process. To be able to distinguish donor and recipient colonies derived from mating mixtures, we used as the recipient *S. solfataricus* PH1, a stable β-galactosidase mutant which is negative in the X-Gal blue reaction (18). Donor NOB8H2, in contrast, exhibits β-galactosidase activity and therefore turns deep blue when incubated with X-Gal. Samples of a mating mixture containing these two strains in a 1:1 ratio were taken at different times within 24 h and plated. As expected, we obtained small white or blue colonies and large white colonies. As judged by DNA analysis of several representatives, all clones derived from the smaller colonies harbored the plasmid and were either *S. solfataricus* transcipients or the donor NOB8H2, whereas almost all of the larger white colonies were *S. solfataricus* cells not containing pNOB8 (not shown). Because of the low plating efficiency of the donor strain and the transcipients (<10%; see also above), we could only estimate the frequency and the kinetics of the transfer process by monitoring the fate of the nonconjugated recipient cells, i.e., by counting the large white colonies. Already within

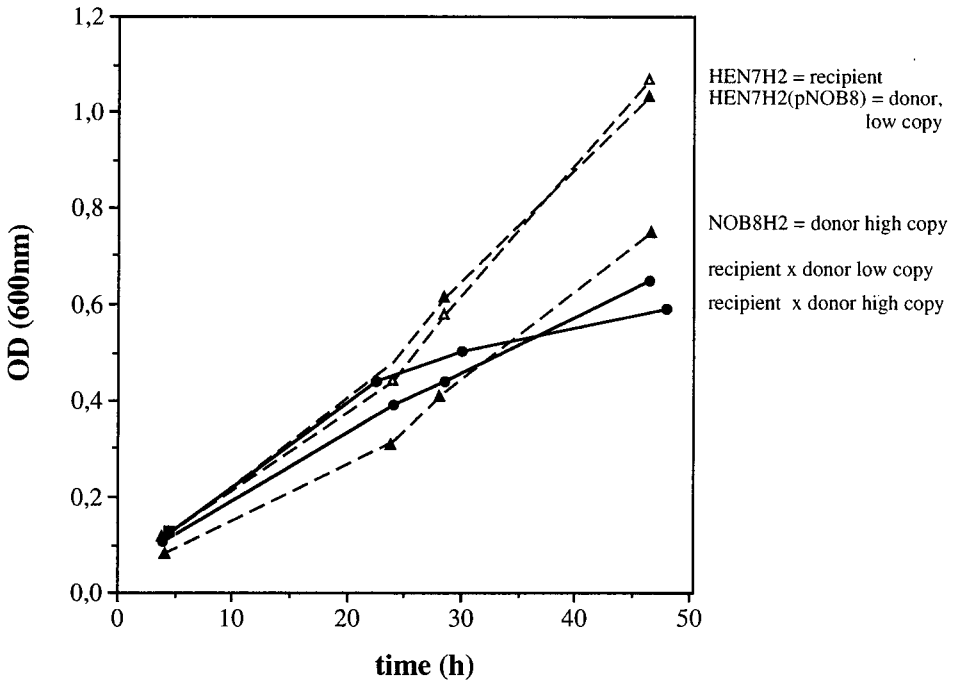

FIG. 4. Growth of recipient strain *S. islandicus* HEN7H2 and donor strains containing the pNOB8 plasmid in different amounts [HEN7H2(pNOB8), low-copy-number donor; NOB8H2(pNOB8), high-copy-number donor] compared with growth of corresponding mating mixtures (donor/recipient cell ratio, 1:1,000). OD, optical density.

the first 3 h a decrease in the number of these colonies was observed, and after 24 h <10% of the original count was recovered on the plate (Fig. 6). Assuming that all cells survive, this would mean that under the conditions chosen around 90% of the recipients had been involved in the mating process within 24 h.

Microscopic analysis of the mating process. In order to better understand the nature of the process that mediates dissemination of pNOB8 in liquid culture, mixtures of donor and recipient cells were analyzed under the light microscope. Since the cells of different *Sulfolobus* strains cannot easily be distinguished by size and/or morphology, we marked them with different DNA-intercalating fluorescent dyes prior to mixing. In the experiments whose results are shown in Fig. 7, the donor strain, NOB8H2, had been stained with Chromomycin A3 (orange-brown) and the recipient, *S. islandicus* HEN7H2, had been stained with Hoechst 33258 (green-blue) prior to mating of the two strains in a 1:1 ratio. The staining was stable for at least 1 h under the growth conditions; however, analysis with the fluorescence microscope had to be performed quickly, since exposure to UV light resulted in rapid bleaching, especially of Chromomycin A3.

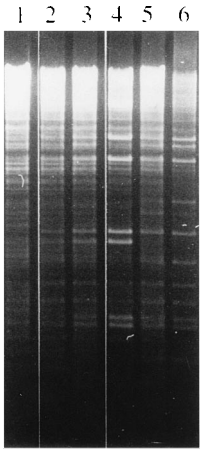

FIG. 5. *Bam*HI digest of total DNAs of *S. islandicus* HEN7H2 (lane 1), HEN7H2(pNOB8) (lanes 2 and 3), and the corresponding mating mixture whose growth is shown in Fig. 4 (lanes 4 to 6). The DNAs were prepared after 2 (lanes 1, 2, and 4), 5 (lane 5), and 7 (lanes 3 and 6) days of cultivation.

Two minutes after the two strains were mixed, we already observed pairs and larger aggregates of differently stained cells that were easily distinguishable from lumps or pairs of cells of a single kind, probably intermediates of cell division, by their difference in color (Fig. 7). Mating pairs were either in close contact or attached to each other, as manifested by the fact that they moved as a pair under the influence of Brownian motion when they had not been fixed in an agarose bed. Such mating pairs and groups of differently stained cells were found in rising numbers over the observation period.

For controls, we mixed two differently stained aliquots of cells of each of the two strains (i.e., donor × donor and recipient × recipient) in order to find out whether they also form pairs under these conditions and how often pseudo-mating pairs might be seen by chance when two cells lie close together. Table 2 shows a quantitative analysis of cell pairs (larger aggregates were not considered) observed 35 min after the two differently stained partners were mixed. Whereas pairs of one kind were not formed or found only in negligible numbers in recipient mixtures (*S. islandicus* HEN7H2 or *S. solfataricus* PH1), strains which harbor the pNOB8 plasmid, i.e., NOB8H2 and HEN7H2(pNOB8), seemed to be able to form such cell pairs, although significantly less frequently than donor-recipient mixtures.

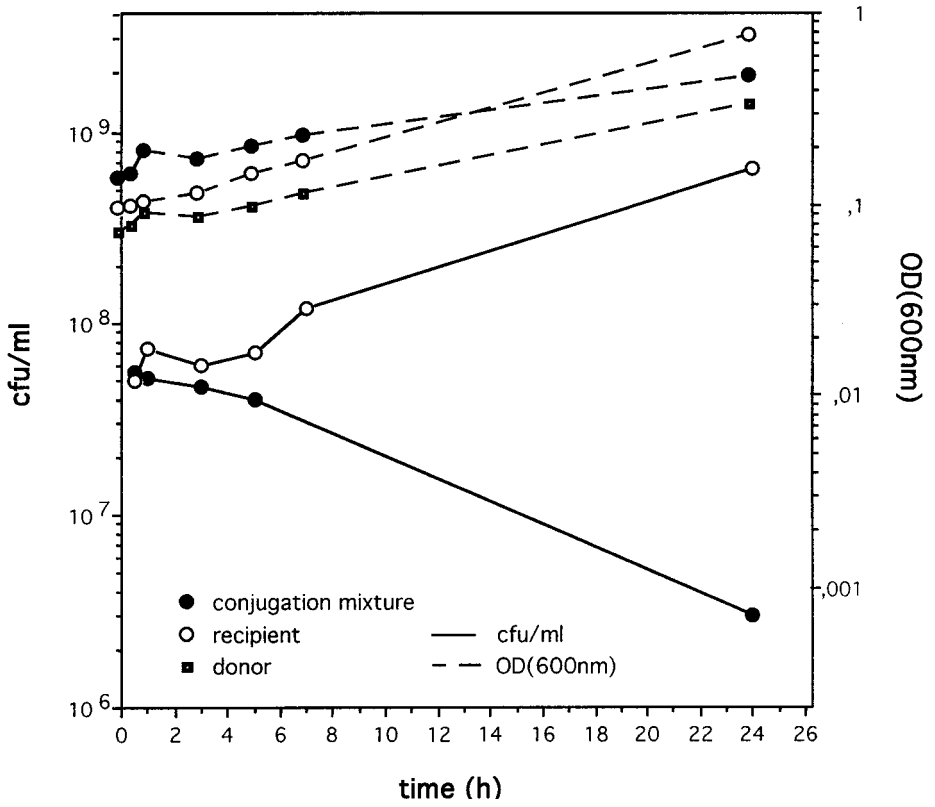

FIG. 6. CFU of recipient strain *S. solfataricus* PH1 (solid circles) obtained from a conjugation mixture (1:1) with NOB8H2 as the donor. Results for a plating control from half of the culture not mixed with the donor are also shown (open circles). - - - -, growth of the corresponding cultures and of donor NOB8H2. OD, optical density.

Electron microscopy of the donor strain (NOB8H2) showed that many of the cells contain cellular appendages probably still covered by the S-layer, which have never been observed in the recipient strains (Fig. 8) and which are likely to belong to the conjugation apparatus. Similar appendages were also observed by scanning electron microscopy (Fig. 9). Because of their dimensions and their irregular appearance, these structures were judged to be neither pili nor flagella. In scanning electron micrographs of conjugation mixtures taken 10 and 30 min after the start of mating, many aggregates of up to about 30 cells were found, sometimes exceeding the number of remaining single cells. In some of these aggregates, intercellular bridges clearly connected two or more cells (Fig. 9).

Variants of pNOB8. We often observed a variation in the restriction fragment pattern of plasmid preparations from cultures of mating mixtures as well as from the original donor strain (Fig. 10). In most of these cases, the original, or wild-type (wt), pattern of pNOB8 was still present, but several fragments occurred in substoichiometric amounts, as if deletion variants were frequently formed (Fig. 10, lanes 2, 3, 6 to 8, 10, and 11). Sometimes new fragments appeared, usually at the expense of others (Fig. 10, lanes 4 and 9; Fig. 1, H3 lane e; and Fig. 2, lanes 4+ and K2). These additional fragments cross-reacted in Southern analysis with the wt pNOB8 DNA (not shown). One type of deletion variant found in several transcipients lacked at least two *Bam*HI fragments, i.e., around 8 kb in total (Fig. 3, lanes 1 and 3, and Fig. 10, lanes 2 and 3). From further analysis of this variant using different restriction digests and suitable Southern hybridizations, we conclude that the missing two *Bam*HI fragments are neighboring in the wt plasmid. The fact that two *Bam*HI bands disappeared without a concomitant shift of other bands suggested that the deletion covered the two neighboring fragments quite accurately. This was confirmed by comparing the pattern of the deletion variant with that of the wt plasmid after cutting with different restriction enzymes. When plasmids were cut with *Eco*RV or *Hin*dIII, shifts and losses of fragments resulting from a loss of approximately 8 kb in the plasmid variant were observed (not shown). Since this deletion variant was shown to propagate in conjugation mixtures (Fig. 4 and 5), we assume that the region comprising these two *Bam*HI fragments is not essential for replication and/or conjugative transfer of the plasmid.

Transformation of pNOB8. Another means of showing spreading of the conjugative plasmid in a culture was to transform *S. solfataricus* with the purified plasmid. Conditions for DNA uptake via electroporation had been established for this strain by transfecting it with the DNA of virus SSV1 (17). Two preparations of pNOB8 were used, one showing the original wt pattern (Fig. 11A, lane 1) and the other apparently a mixture of the wt plasmid and an insertion variant, since an additional fragment appeared substoichiometrically at the expense of a smaller fragment of the wt pattern (Fig. 11B, lane 1). After electroporation, the cells were grown for 30 h and total DNA was prepared and analyzed. In both cases, the plasmid was found in very high average copy numbers in the cells that had been subjected to transformation (lanes 2 in Fig. 11 represent total DNA). Since the frequency of transformation determined in the transfection experiments with SSV1 DNA is only 10^{-4} to 10^{-5}, the relatively few transformants obtained by electroporation must have served as donors for further spread of the plasmid via conjugation. Accordingly, total DNA prepared shortly after transformation, i.e., before the conjugative

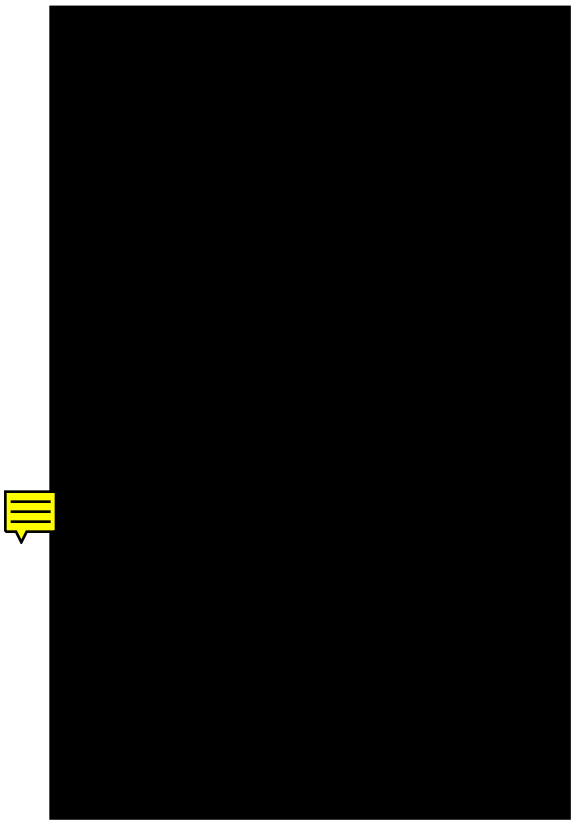

FIG. 7. Mating pairs and cell aggregates observed in a mating mixture (1:1) of NOB8H2 (donor), stained with Chromomycin A3 (orange-brown), and *S. islandicus* HEN7H2 (recipient), stained with Hoechst no. 33258 (green-blue). The cells were viewed under fluorescent light (left panels) and with Numarsky phase-contrast (right panels).

spread, showed a low average copy number of pNOB8, probably representing mainly the plasmid in the primary transformants (not shown). Furthermore, growth of the cells subjected to electroporation with pNOB8 was retarded for several days, and plating of these cultures after 2 days gave rise to only tiny colonies, as observed in cultures of conjugation mixtures, suggesting that the plasmid had spread to all cells. In the experiment whose results are shown in Fig. 11B, the stoichiometry of the restriction fragments of the resulting plasmid has changed compared with that of the input plasmid, as if a deletion variant resembling that described above plus a smaller amount of a second variant had outcompeted the wt plasmid.

TABLE 2. Mating pairs observed after mixing of differently stained partners

Mixture	No. of pairs/field[a]
NOB8H2(pNOB8) × *S. islandicus* HEN7H2	20
NOB8H2(pNOB8) × *S. solfataricus* PH1	14
NOB8H2(pNOB8) × NOb8H2(pNOb8)	7
S. islandicus HEN7H2(pNOb8) × *S. islandicus* HEN7H2(pNOB8)	3
S. islandicus HEN7H2 × *S. islandicus* HEN7H2	0
S. solfataricus PH1 × *S. solfataricus* PH1	2

[a] Pairs of cells of different colors were counted 35 min after two strains were mixed under culture conditions. Each result is the average for 10 analyzed microscopic fields. (One cell per microscopic field = 2.7×10^6 cells per ml.)

DISCUSSION

pNOB8 is the first archaeal plasmid for which horizontal spreading has been demonstrated. Despite its relatively large size (45 kb), the plasmid replicates to unusually high copy numbers both in the original donor strain and in mating mixtures in which different *Sulfolobus* species can act as recipients. We have proved the transfer of the plasmid from the original host, NOB8H2, to cells of *S. solfataricus* and *S. islandicus* by isolating transcipients via single colonies. Although the exact mechanism of this transfer is still unknown, several lines of evidence support a conjugation-like process as follows.

(i) Transmission of the plasmid depended on the presence of donor cells, whereas donor filtrates were inactive, suggesting that the transfer does not involve a virus. Virus particles were not observed with the electron microscope, nor were such particles isolated from culture supernatants. Furthermore, plating of donor cells or supernatants from donor cultures on lawns of recipients did not yield plaques, although the spread of the plasmid markedly retarded cell growth and although the plaque assay works well for two different *Sulfolobus* viruses (SSV1 of *S. solfataricus* [17] and SIRV1 of *S. islandicus* [25]).

(ii) Naked DNA is unstable under the growth conditions of *Sulfolobus* cells, making a natural transformation involving free DNA improbable.

(iii) The transfer appears to occur rapidly and/or at a high frequency, since no recipient cells devoid of pNOB8 were recovered from mating mixtures (1:1,000) after 2 days. However, so far we have not attempted to determine the velocity and frequency of the transfer process, because donor and transcipient cells plate only with low efficiency.

(iv) Transformation of a recipient (*S. solfataricus* P1) with purified plasmid DNA resulted in the spread of the plasmid through the entire culture, suggesting that the relatively few transformants obtained via electroporation must have served as donors for further conjugative transfer of the plasmid.

(v) The ability of transcipients and transformants to serve as donors for the spread of pNOB8 strongly implies that the conjugation system is encoded on the plasmid itself and not on the chromosome of the donor strain.

(vi) In the fluorescence microscope, de novo cell-to-cell contacts were observed to form rapidly after differently stained donor and recipient partners were mixed, whereas almost no such aggregates were seen in the absence of plasmid (i.e., recipient × recipient). Scanning electron micrographs of mating mixtures revealed many cell aggregates made up of 2 to 30 cells. Sometimes cytoplasmic bridges appeared to connect two or more cells, whereas thinner connecting structures such as pili were not observed.

We have found the plasmid in high copy number, between 20 and 40 per chromosome, in the original donor strain and in mating mixtures, whereas isolates obtained by plating mating mixtures exhibited copy numbers as low as 1 to 5. Since the plating efficiency of transcipients from mating mixtures was low (<10%), it seems possible that we have selected only the fraction of the transcipients which contained the plasmid in lower and thus less deleterious copy numbers. Another cryptic plasmid usually harbored in high copy number by one of the recipient types (*S. islandicus* HEN7H2) was found to be equally reduced in such isolates. We have observed that the average copy number of pNOB8 in a mating mixture rises when we use a low-copy-number transcipient as the donor and that it falls again after several days (Fig. 5). The natural copy number of the plasmid might therefore be low, and conjugation strongly induces replication until the control system in the transcipients is established. Consequently, it is possible that only that frac-

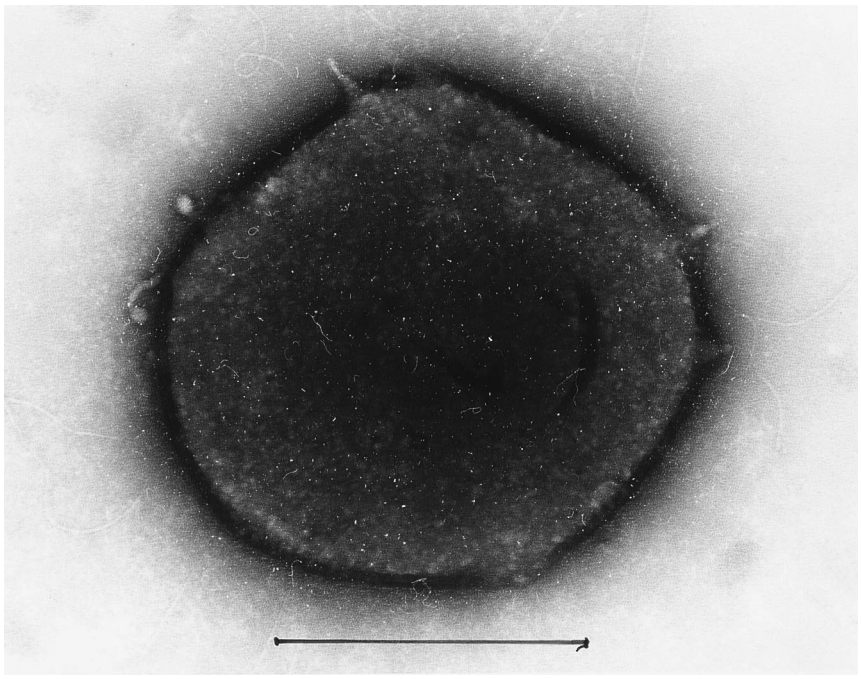

FIG. 8. Electron micrograph of a cell of NOB8H2 exhibiting cellular appendages and protruding structures (pili are also seen). The cell was negatively stained with uranyl acetate. Bar = 1 μm.

tion of the transcipient progeny in which the plasmid has already been downregulated might produce visible colonies on the plate. However, this model does not explain why the copy number in the original donor strain, NOB8H2, is very high, whereas the progeny of this strain isolated from mating mixtures contain the plasmid in low copy number. Alternatively, other, yet-unknown stimuli might trigger a strong replication of the plasmid. In this regard, it is noteworthy that we have observed a strong replication of pNOB8 in cultures of low-copy-number transcipients inoculated from glycerol stocks (not shown).

The high copy number of the plasmid in mating mixtures and the resulting growth retardation of these cultures suggest that the mechanism of spread in certain respects may resemble that of viruses more than that of known bacterial conjugative plasmids. The existence of appendages in the original host of pNOB8 and of intercellular bridges in mating mixtures which might be formed by such appendages could be taken to indicate that conjugation is mediated by some sort of fusion similar to that suggested for the mating system of *H. volcanii* (20). In that system, the gene transfer involves bidirectional exchange of chromosomal genes and the cotransfer of selectable recombinant plasmids. The extent and direction of gene transfer in the *Sulfolobus* system as well as the mechanisms by which cell-to-cell contacts are formed and by which the DNA is transferred await further investigation.

Sequence analysis of the plasmid might give further insight into the factors involved in mating and their relation to those of the bacterial systems. The large size of pNOB8 suggests that a rather complex system could be encoded on the plasmid.

An unusual feature of pNOB8 is its extensive genetic variation, especially caused by formation of deletion variants. One such variant that was grown from a single transcipient colony was still able to propagate in mating mixtures. Other deletion variants, however, could be only passively replicated and/or transmitted via conjugation, since they were found in plasmid

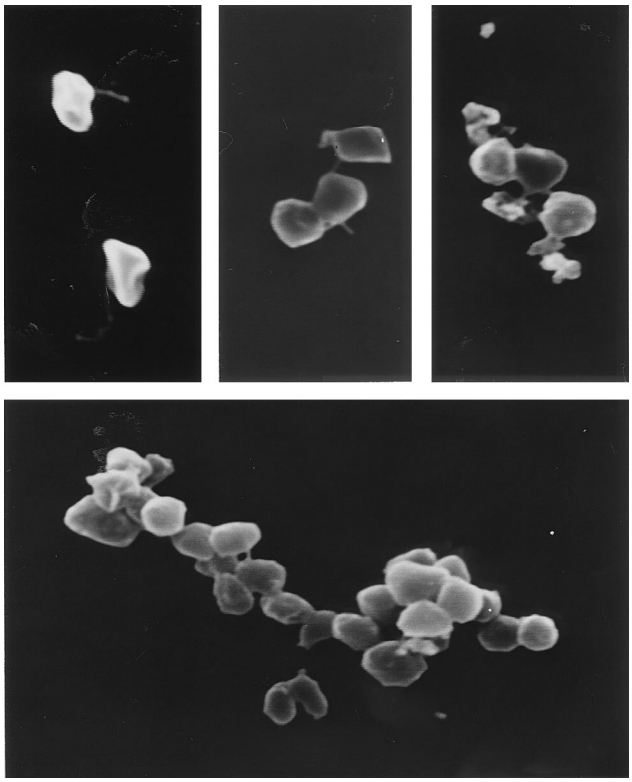

FIG. 9. Scanning electron micrographs of donor strain NOB8H2 (upper left) and 1:1 mating mixtures of NOB8H2 × *S. solfataricus*, showing cellular appendages of the donor strain and cell aggregates and intercellular bridges in the mating mixture.

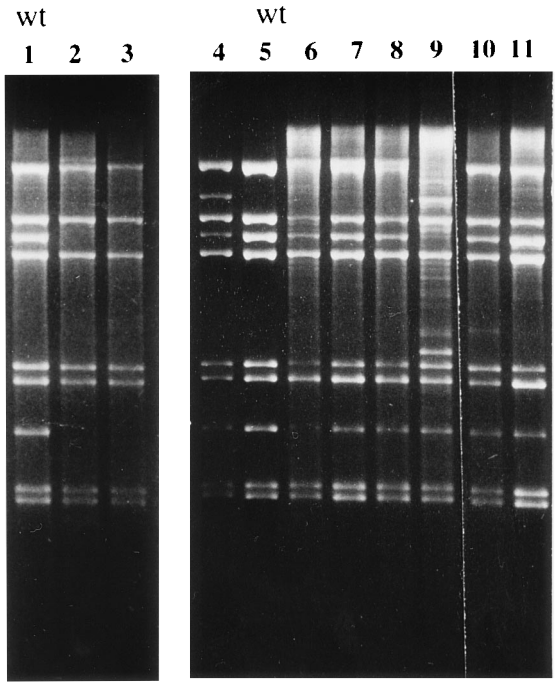

FIG. 10. Total DNAs (lanes 1 to 3 and 6 to 11) and cccDNAs (lanes 4 and 5) obtained from different cultures of NOB8H2 (lanes 4 to 6, 9, and 11) or from conjugation mixtures (lanes 1 to 3, 7, 8, and 10) that show variation from the original (wt) pattern (as seen in lanes 1 and 5).

preparations that still contained the wt plasmid. The high copy number of pNOB8, which retards growth of its host, might favor the formation of smaller, less deleterious derivatives. In contrast, some other variants seem to be enlarged compared with the original form of pNOB8. They could be forms that have gained insertion elements (as found in *S. solfataricus* [18]) or prime plasmids containing parts of the host chromosome. Whether pNOB8 is capable of recombination with the chromosome is still unknown, however.

Because of its efficient spread and relatively broad host range, the pNOB8 plasmid should prove useful for the development of a genetic system for *Sulfolobus* spp. We and others have previously demonstrated the uptake of viral and plasmid DNAs by *S. solfataricus* via electroporation (17, 25). Recombinant derivatives of these genetic elements that are still able to replicate or propagate autonomously have so far not been identified. Preliminary experiments with pNOB8, however, have shown that the plasmid can be manipulated at several sites and that transformants are easily detectable even in the absence of a selectable genetic marker because of the efficient spread of the plasmid through the culture (unpublished data). The plasmid should therefore prove useful for the construction of transformation vectors that will be easily transferable to various *Sulfolobus* strains by conjugation and that should serve for efficient expression because of their high copy number.

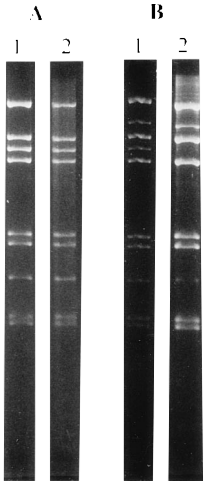

FIG. 11. *Bam*HI digest of input and output DNAs of two transformation experiments (A and B) with pNOB8. Lanes 1, the plasmid preparations of which 200 ng each was used for electroporation of *S. solfataricus* P1; lanes 2, 2 μg of total DNA prepared from the whole-cell mixture 30 h after electroporation.

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REFERENCES

1. **Brisson-Noel, A., M. Arthur, and P. Courvalin.** 1988. Evidence for natural gene transfer from gram-positive cocci to *Escherichia coli*. J. Bacteriol. **170:** 1739–1745.
2. **Brock, T. D., K. M. Brock, R. T. Belly, and R. L. Weiss.** 1972. *Sulfolobus*: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. Arch. Microbiol. **84:**54–68.
3. **Clewell, D. B. (ed.).** 1993. Bacterial conjugation. Plenum Press, New York.
4. **Davies, J.** 1994. Inactivation of antibiotics and the dissemination of resistance genes. Science **264:**375–382.
5. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
6. **Frost, L. S., K. Ippen-Ihler, and R. A. Skurray.** 1994. Analysis of the sequence and gene products of the transfer region of the F sex factor. Microbiol. Rev. **58:**162–210.
7. **Grogan, D., P. Palm, and W. Zillig.** 1990. Isolate B12, which harbours a virus-like element, represents a new species of the archaebacterial genus *Sulfolobus*, *Sulfolobus shibatae*, sp. nov. Arch. Microbiol. **154:**594–599.
8. **Heinemann, J. A., and G. F. Sprague, Jr.** 1989. Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeast. Nature (London) **340:**205–209.
9. **Kondo, S., A. Yamagishi, and T. Oshima.** 1993. A physical map of the sulfur-dependent archaebacterium *Sulfolobus acidocaldarius* 7 chromosome. J. Bacteriol. **175:**1532–1536.
10. **Lessl, M., and E. Lanka.** 1994. Common mechanism in bacterial conjugation and Ti-mediated T-DNA transfer to plant cells. Cell **77:**321–324.
11. **Mazodier, P., and J. Davies.** 1991. Gene transfer between distantly related bacteria. Annu. Rev. Genet. **25:**147–171.
12. **Meile, L., P. Abendschein, and T. Leisinger.** 1990. Transduction in the archaebacterium *Methanobacterium thermoautotrophicum* Marburg. J. Bacteriol. **172:**3507–3508.
13. **Mevarech, M., and R. Werczberger.** 1985. Genetic transfer in *Halobacterium volcanii*. J. Bacteriol. **162:**461–462.
14. **Rosenshine, I., and M. Mevarech.** 1991. The kinetics of the genetic exchange process in *Halobacterium volcanii* mating, p. 265–270. *In* R. Rodriguez-Valera (ed.), General and applied aspects of halophilic microorganisms. Plenum Press, New York.
15. **Rosenshine, I., R. Tchelet, and M. Mevarech.** 1989. The mechanism of DNA transfer in the mating system of an archaebacterium. Science **245:**1387–1389.
16. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
17. **Schleper, C., K. Kubo, and W. Zillig.** 1992. The particle SSV1 from the extremely thermophilic archaeon *Sulfolobus* is a virus: demonstration of infectivity and of transfection with viral DNA. Proc. Natl. Acad. Sci. USA **89:**7645–7649.
18. **Schleper, C., R. Roeder, T. Singer, and W. Zillig.** 1994. An insertion element of the extremely thermophilic archaeon *Sulfolobus solfataricus* transposes into the endogenous β-galactosidase gene. Mol. Gen. Genet. **243:**91–96.
19. **Sikorski, R. S., W. Michaud, H. L. Levin, J. D. Boeke, and P. Hieter.** 1990. Trans-kingdom promiscuity. Nature (London) **345:**581–582.
20. **Tchelet, R., and M. Mevarech.** 1993. Interspecies genetic transfer in halophilic archaebacteria. Syst. Appl. Microbiol. **16:**578–581.

21. **Trieu-Cuot, P., C. Carlier, P. Martin, and P. Courvalin.** 1987. Plasmid transfer by conjugation from *Escherichia coli* to Gram-positive bacteria. FEMS Microbiol. Lett. **48:**289–294.
22. **Willets, N., and R. Skurray.** 1980. The conjugation system of F-like plasmids. Annu. Rev. Genet. **14:**41–76.
23. **Willets, N., and B. Wilkins.** 1984. Processing of plasmid DNA during bacterial conjugation. Microbiol. Rev. **48:**24–41.
24. **Woese, C. R., O. Kandler, and M. L. Wheelis.** 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria and Eucarya. Proc. Natl. Acad. Sci. USA **87:**4576–4579.
25. **Zillig, W., A. Kletzin, C. Schleper, I. Holz, D. Janekovic, J. Hain, M. Lanzendörfer, and J. K. Kristjansson.** 1993. Screening for *Sulfolobales*, their plasmids and their viruses in Icelandic solfataras. Syst. Appl. Microbiol. **16:**609–628.
26. **Zillig, W., K. O. Stetter, S. Wunderl, W. Schulz, H. Priess, and I. Scholz.** 1980. The *Sulfolobus*-"Caldariella" group: taxonomy on the basis of the structure of DNA-dependent RNA polymerases. Arch. Microbiol. **125:**259–269.