

Heterogeneity of Small Plasmids from Halophilic Archaea

A. S. AKHMANOVA,† V. K. KAGRAMANOVA, AND A. S. MANKIN‡*

*Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University,
119899 Moscow, Russia*

Received 22 September 1992/Accepted 4 December 1992

Small multicopy plasmids in three strains of halophilic archaea, SB3, GRB, and GN101, were found to be present in a cell as a population of related but not identical sequences. Two types of heterogeneity were observed: macroheterogeneity, represented by two major plasmid sequence versions homologous to each other by 80%, and microheterogeneity, in which individual plasmids differ by one or a few nucleotide substitutions.

Plasmids are self-replicating extrachromosomal genetic elements present in a cell in multiple copies. Usually, all copies of the same plasmid have the same nucleotide sequence. Similar but not identical plasmids generated by mutation are normally incompatible and rapidly segregate so that eventually only one plasmid variant is found in the cell culture (for reviews, see references 3 and 17).

The presence of extrachromosomal circular DNA is characteristic of many species from the domain *Archaea* (23). Thus, several strains of halophilic archaea contain plasmids that range in size from 30 to 100 kbp and exist in a relatively low number of copies per cell (1, 4-6, 11, 18, 19). In addition, a small plasmid (about 1.7 kbp) with a high copy number has been found in three halobacterial isolates, SB3, GRB, and GN101 (5). The small plasmids from these three strains are closely related, as originally revealed by hybridization analysis (5) and later by sequencing (7-9, 14), but share no detectable homology either with the chromosome of the host strains or with DNAs of other halobacteria. The existence of a small amount of a single-stranded plasmid form in the cell may represent an intermediate generated by rolling-circle replication (22).

While cloning and sequencing a small plasmid from the SB3 strain, we observed that some of the presumably identical recombinant plasmids isolated from the transformed *Escherichia coli* colonies in fact differed from each other by a few base changes (13). This unexpected phenomenon was examined in detail, and in this report we present evidence that the small plasmids in a halobacterial cell do not have a unique sequence but, instead, exist as a population of closely related sequences. Two main types of heterogeneity were detected: microheterogeneity, in which individual plasmids differ from each other by one or a few nucleotide substitutions, and macroheterogeneity, represented by two major plasmid versions exhibiting about 80% homology.

MATERIALS AND METHODS

Strains, media, and enzymes. *Halobacterium* strains SB3, GRB, and GN101 (5) were kindly provided by F. Pfeifer and

N. Hackett. Cells were grown in liquid complex media (2) at 37°C with constant agitation or on 1.5% agar plates prepared with the same media diluted with one-fifth of a volume of water. *E. coli* plasmids were propagated in strains XL1 and DH5 α . Cultures of *E. coli* cells were grown in 2 \times YT medium in the presence of 50 μ g of ampicillin per ml (20).

All of the enzymes used in this work were from Bio-preparat (Vilnius, Lithuania).

Plasmid isolation and DNA analyses. Halobacterial colonies were isolated by a spreading technique (20), and cell cultures were grown from individual clones. Cells were harvested at the early stationary phase (A_{550} , ~1.2), and total plasmids were isolated from cultures by a conventional NaOH-sodium dodecyl sulfate procedure (20) with omission of lysozyme from the lysis buffer. RNA was removed by RNase treatment and polyethylene glycol precipitation (10), and the supercoiled form of a 1.75-kbp plasmid was further purified from a 0.8% agarose gel.

Recovered plasmid DNA was subjected to restriction analyses, and the resulting DNA fragments were resolved in agarose or acrylamide gels. Gels were stained with ethidium bromide, and the relative intensity of DNA bands was quantified with an Ultrascan (LKB) scanner.

Cloning and sequencing of halobacterial plasmids. Genetic engineering manipulations were performed as described elsewhere (20).

A small plasmid from *Halobacterium* strain SB3 was linearized by *SalI* hydrolysis and ligated with the pUC19 vector, which was cut with the same enzyme. The ligation mixture was transformed into *E. coli* DH5 α cells. Recombinant plasmids were isolated from several individual colonies and analyzed by digestion with the *Sau3A*, *TaqI*, *MspI*, *Eco47I*, or *BspRI* restriction enzyme, which allowed discrimination of pHSB1-pUC19 from pHSB2-pUC19 (see Results).

For sequencing, the insert of one of the pHSB2-pUC19 recombinant clones was excised and digested with *MspI* or *TaqI* and subfragments were cloned into the *AccI* site of the M13 mp9 vector. The nucleotide sequence of both strands of pHSB2 was determined from the resultant M13 recombinant bacteriophages or from the original pHSB2-pUC19 clone by the dideoxy-chain termination technique (21). Analysis of the sequencing results was done with the DNA Master program (16).

Nucleotide sequence accession numbers. The GenBank accession numbers for the pHSB1 and pHSB2 sequences discussed here are X07128 and X66324, respectively.

* Corresponding author.

† Present address: Department of Molecular and Developmental Genetics, Catholic University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands.

‡ Present address: Department of Medicinal Chemistry and Pharmacognosy, m/c 781, University of Illinois at Chicago, Chicago, IL 60680-6998.

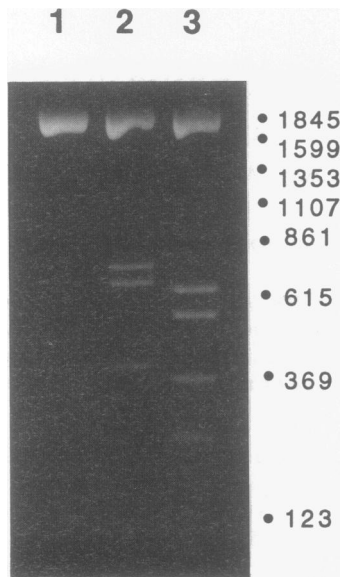


FIG. 1. Macroheterogeneity of the small plasmid from *Halobacterium* strain SB3. A 1.75-kbp plasmid isolated from a culture grown from a single colony and further purified in agarose gel as described in Materials and Methods was hydrolyzed with restriction enzyme(s) *SalI* (lane 1), *Sau3A* (lane 2), or *HindIII-Eco47I* (lane 3) and analyzed in a 2% agarose gel. Mobility of marker DNA fragments and their molecular sizes (in base pairs) are indicated to the right of the gel.

RESULTS AND DISCUSSION

Macroheterogeneity of plasmid pHSB. The heterogeneity of small plasmid pHSB in halobacterial strain SB3 was first noticed during its sequencing (13). In those experiments, restriction fragments of pHSB were subcloned into vectors which were propagated in *E. coli* cells. In this shotgun sequencing approach, the same fragment is usually sequenced several times from individual isolates. Normally, this would yield identical sequences. However, this was not the case for pHSB, since we repeatedly noticed that DNA fragments which were presumably identical occasionally exhibited slightly different nucleotide sequences. We were able to show that at least one of the nucleotide variations was not a result of cloning in *E. coli* but was already present in the original pHSB preparation (13).

To study this phenomenon in more detail, we analyzed the possible heterogeneity of the 1.75-kbp pHSB plasmid isolated from the individual colonies of *Halobacterium* strain SB3. Figure 1 represents restriction patterns generated by digestion of the plasmid with different restriction enzymes. While hydrolysis with *SalI* apparently linearizes pHSB into a single 1.75-kbp DNA fragment, digestion with *Sau3A* generates the main linear form and three additional bands (also comprising about 1.7 kbp). The same result was observed with a *HindIII-Eco47I* double digest in which the linear form is accompanied by four additional fragments which together roughly correspond to the full-length plasmid. One possible explanation for these results was that two plasmids, both with a size of ca. 1.75 kbp but differing in sequence, coexist in the cell. Specifically, one of the plasmids, designated pHSB1, would correspond to the linear plasmid form in lanes 2 and 3 in Fig. 1. The other, pHSB2, should have several *Sau3A* and *Eco47I* sites and would

account for shorter bands in these lanes. The approximate ratio of pHSB1 to pHSB2, as estimated from the band intensities on ethidium bromide-stained gels, was 2.5:1. This ratio did not exhibit noticeable variation between cultures grown from 10 individual colonies.

To test the hypothesis of the coexistence of two plasmids in a cell, the 1.75-kbp *SalI* band (Fig. 1, lane 1) was cloned into the pUC19 vector. Sequence analysis of individual recombinant clones indeed revealed the presence of two plasmid forms, pHSB1 and pHSB2. Deduced sequences (Fig. 2) substantiated the above interpretation of restriction analysis. The 1,736-bp pHSB1 primary structure, corresponding to the pHSB sequence reported previously (7, 14), had unique *SalI*, *HindIII*, and *Sau3A* sites and no *Eco47I* site (and thus accounts for the linear plasmid bands in Fig. 1). In contrast, 1,781-bp plasmid pHSB2 had no *HindIII*, one *SalI*, three *Sau3A*, and five *Eco47I* sites, accounting for the shorter bands in the *Sau3A* and *HindIII-Eco47I* digests (only four pHSB2 bands are seen in the *HindIII-Eco47I* digest because the fifth fragment is only 11 bp long). Despite these differences, the overall degree of homology between plasmids pHSB1 and pHSB2 is very high (about 80%), so that their sequences can be readily aligned (Fig. 2). The most conserved part of the plasmids corresponds to a 316-codon open reading frame starting at the ATG at position 671 (pHSB1) or 716 (pHSB2). Interestingly, of 140 nucleotide substitutions within this open reading frame, most either do not change the encoded amino acid (66 of 110 mutated codons) or lead to conservative amino acid substitutions (another 16 codons), which suggests that the structure and function of the encoded protein are conserved. The putative protein, whose gene accounts for 55% of the plasmid length, is likely involved in plasmid maintenance, since sequences homologous to its gene are missing in the DNAs of strains which do not contain small multicopy plasmids (5) and characteristic sequence motifs of REP proteins mediating plasmid rolling-circle replication are present (12).

Most of the differences between the pHSB1 and pHSB2 plasmids are clustered in the region between positions 300 and 550 (pHSB1 numbering), where a substantial number of nucleotide substitutions, insertions, and deletions are located. It is not known whether this region is of any functional significance or whether functional sites, including those involved in the control of plasmid replication, are confined to the more conserved plasmid sequences.

The fact that pHSB1 and pHSB2 plasmid variants are found in the cultures grown from the individual halobacterial colonies strongly argues in favor of their coexistence in an individual cell. Difference between pHSB1 and pHSB2 sequences might be significant enough to explain their compatibility (especially if sequences responsible for replication control are located within the less conserved region). Alternatively, pHSB1 and pHSB2 might be generally incompatible but the process of their segregation is counterbalanced by some factors, such as plasmid exchange between cells within the population or selective pressure for the simultaneous presence of both plasmids. The latter, however, seems unlikely, since we were able to transform *Halobacterium halobium* R₁, which lacks small plasmids, with an individual pHSB2 plasmid which was excised from the pHSB2-pUC19 clone and circularized, and we found that it could be maintained in halobacterial cells in the absence of pHSB1 (data not shown). In addition, a vector based on the pHSB2-equivalent plasmid from strain GRB1 (see below) propagated stably in *H. halobium* (15).

Surprisingly, despite long homologous regions in pHSB1

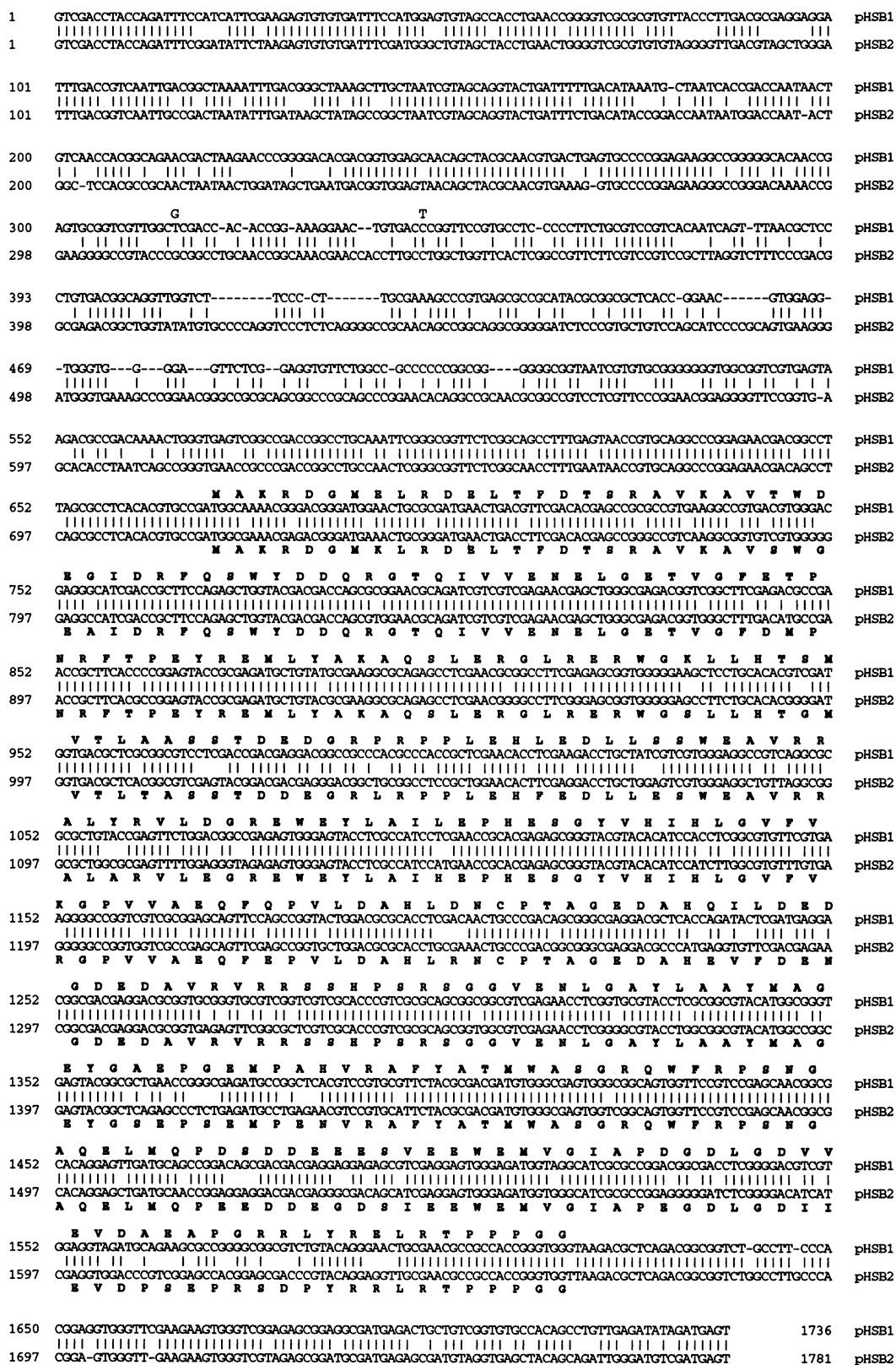


FIG. 2. Alignment of pHSB1 and pHSB2 sequences. Dashes indicate nucleotide deletions; vertical lines indicate nucleotides that are identical in both sequences. Nucleotide substitutions in the *TaqI*⁻ and *MspI*⁻ versions of pHSB1 are indicated above the pHSB1 sequence at positions 315 and 342. Amino acids are shown above (pHSB1) or below (pHSB2) corresponding codons in the conserved open reading frame.

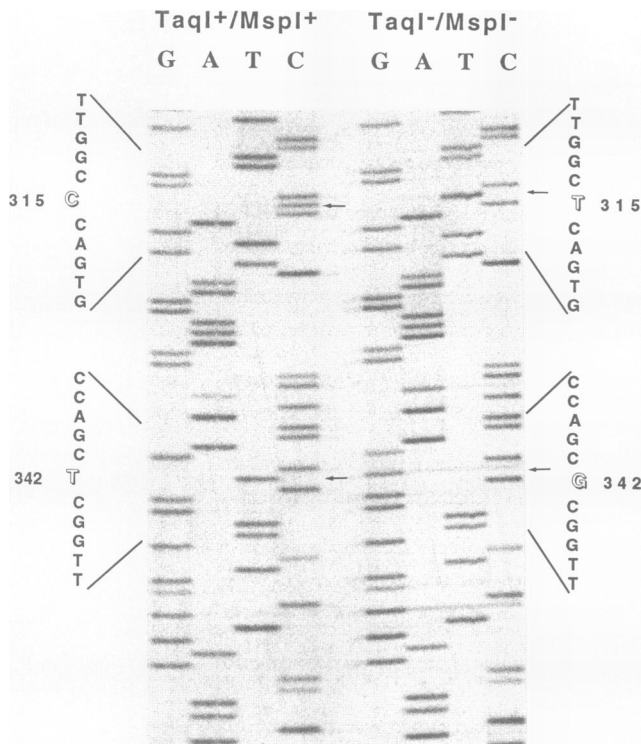


FIG. 3. Nucleotide substitutions at *TaqI* and *MspI* sites of pHSB1 microheterogeneous versions. Portions of sequencing gels of *TaqI*⁺/*MspI*⁺ and *TaqI*⁻/*MspI*⁻ clones are shown. Variable positions are indicated by arrows and numbered. Dideoxy sequencing was performed on double-stranded DNAs of pHSB1-pUC19 recombinant clones.

and pHSB2, restriction analysis of the plasmid isolated from SB3 cells or of more than 30 clones from the pHSB-pUC19 library revealed no recombinants between pHSB1 and pHSB2. This is remarkable, given the high rate of recombination characteristic of several halobacterial strains (4, 18), and suggests that hybrid plasmids are not stable.

Microheterogeneity of plasmid pHSB1. The heterogeneity of pHSB plasmids is not limited to pHSB1 and pHSB2 variants. Another type of sequence variability, microheterogeneity, was revealed by restriction fragment length polymorphism when the collection of pHSB1-pUC19 recombinant clones was screened with restriction enzymes *TaqI* and *MspI*, both of which have numerous cutting sites within the pHSB1 sequence, 17 and 20, respectively. Surprisingly, while some of the recombinant pHSB1-pUC19 plasmids could be cleaved at all of the *TaqI* sites predicted from the pHSB1 and pUC19 sequences (*TaqI*⁺ clones), other plasmids lacked the *TaqI* site at position 315 of pHSB1 (*TaqI*⁻ clones); similarly, only 8 of 10 pHSB1-pUC19 clones analyzed were cleaved at the predicted *MspI* site at position 342 (*MspI*⁻ clones). Notably, this restriction site polymorphism did not result from cloning in *E. coli*, since we have previously shown that *Taq*⁺ and *Taq*⁻ variants of pHSB1 coexist in halobacterial cells (13). Sequencing of the corresponding regions of *TaqI*⁻ and of *MspI*⁻ pHSB1-pUC19 clones revealed T→G and C→T substitutions at positions 315 and 342, respectively, which map to the disrupted restriction sites (Fig. 3). However, no other deviations from the pHSB1 primary structure were found either by restric-

tion analyses with frequently cutting enzymes (*MspI*, *TaqI*, and *BspRI*) or by sequencing of a substantial part (more than one-third) of the plasmid sequence of *TaqI*⁻ and *MspI*⁻ clones. This suggests that *TaqI*⁻ and *MspI*⁻ versions differ from the main variant of pHSB1 by only one or a few base substitutions.

Interestingly, all four possible combinations of *Taq* and *Msp* variations (*TaqI*⁺/*MspI*⁺, *TaqI*⁻/*MspI*⁺, *TaqI*⁺/*MspI*⁻, and *TaqI*⁻/*MspI*⁻) were found in the collection of pHSB1-pUC19 clones analyzed. This indicates the coexistence of all four variants in halobacterial cells and might be explained by recombination between pHSB1 microversions. Note that microheterogeneous sites are only 26 bp apart and thus the rate of recombination should be relatively high to account for their shuffling. Other explanations, such as an unusually high frequency of DNA replication errors at these sites, also cannot be excluded.

Since microvariations of the pHSB1 sequence at positions 315 and 342 have been found because of their fortuitous location within *TaqI* or *MspI* sites, it is possible that other sites of microheterogeneity exist in pHSB1 but escaped detection because they do not coincide with a restriction site. However, it must be noted that polymorphism at neither the other numerous *TaqI* and *MspI* sites nor any of the multiple *BspRI* sites in pHSB1 was observed, suggesting that base substitutions are not randomly scattered along the length of the plasmid but occur at specific positions in the plasmid DNA. Also, we were not able to detect any restriction site polymorphism for plasmid pHSB2 by using enzymes *TaqI*, *MspI*, and *BspRI*.

Heterogeneity of small plasmids from other halobacterial strains. It is noteworthy that small-plasmid heterogeneity is not confined to only one halobacterial strain. We also observed both macro- and microheterogeneity for the plasmid from halobacterial strain GRB. While a reported nucleotide sequence of plasmid pGRB cloned in the *E. coli* vector (4) was almost identical to that of pHSB2, restriction analysis of the small plasmid in strain GRB additionally revealed the presence of a pHSB1-equivalent plasmid variant which was indistinguishable from pHSB1 for several frequently cutting restriction enzymes (data not shown). However, in contrast to the predominance of pHSB1 over pHSB2, the pHSB2 homolog in strain GRB was about five times more abundant than the pHSB1 homolog (Fig. 4), as revealed by scanning of ethidium bromide-stained gels. As in strain SB3, *TaqI*⁺/*TaqI*⁻ and *MspI*⁺/*MspI*⁻ microvariants of the pHSB1-equivalent plasmid coexist in GRB cells.

The small plasmid from a third halobacterial strain, GN101, also exhibits sequence microheterogeneity: the 1.7-kbp plasmids in this strain differ by the presence of either one or two *SaI* sites (data not shown).

Stable maintenance of a heterogeneous plasmid population. Micro- and macroheterogeneity of the small plasmids in halobacteria described in this report is stably maintained. This conclusion is supported by the fact that the same (or very similar) types of heterogeneous plasmids were observed not only in individual colonies of the same strain but also in strains which were isolated from geographically distant locations (France for GRB and the United States for SB3 [5]). Assuming that these strains have been evolving separately from each other for a considerable time (which is reflected in a difference in the spectra of the large plasmids [5]), it is remarkable that during this period only the ratios between major variants of the small plasmids have changed (if it is not due just to difference in the genotypes of the host

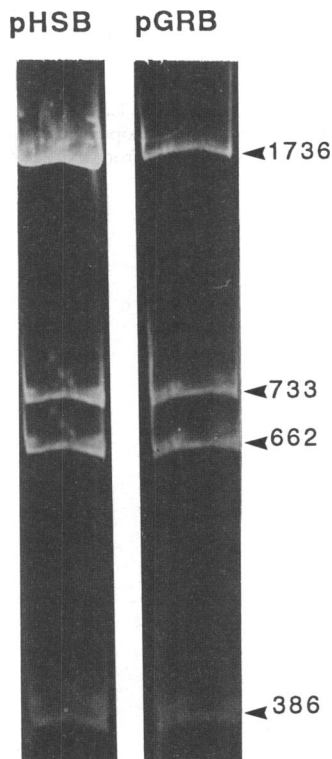


FIG. 4. Difference in the ratios of the major variants of the 1.75-kbp plasmid between strains SB3 and GRB. *Sau*3A digests of the small plasmids from the two strains were analyzed in a non-denaturing 5% polyacrylamide gel. The 1,736-bp band represents a linear form of pHSB1, which contains one *Sau*3A site. Three shorter bands (733, 662, and 386 bp) correspond to pHSB2 (three *Sau*3A sites). Note the difference in relative intensity of the 1,736-bp band compared with the shorter bands between the two strains. The numbers on the right are molecular sizes in base pairs.

cells) while all of the macro- and microheterogeneous plasmid versions have been preserved.

A question that remains unanswered is whether the same mechanism accounts for intracellular populational stability of macro- and microheterogeneity of the small halobacterial plasmids. On the one hand, the fact that both types of heterogeneity are found in the same cell is intriguing and can hardly be ascribed to pure coincidence. Thus, a common mechanism like, for example, efficient horizontal transfer of the plasmids within the cell population might explain the observed phenomena. On the other hand, it appears that while plasmid macrovariants do not recombine with each other, the microversions presumably do. This might imply different mechanisms of maintenance of macro- or microvariants, since efficient recombination can significantly slow down or even completely abolish plasmid segregation.

Sequence heterogeneity of the small plasmids within the same cell, found for extremely halophilic archaea, has not been described for plasmids of other organisms. Our understanding of archaeal molecular biology is insufficient to permit us to distinguish whether our observations reflect a specific feature of halobacteria or represent a more general phenomenon. Recently developed vector transformation systems for halobacteria may help in unravelling some of the unexplained peculiarities of plasmid maintenance in archaea.

ACKNOWLEDGMENTS

Thanks are due to F. Pfeifer and N. Hackett for halobacterial strains and to D. W. Mount and B. Conrad for providing the DNA Master computer program. We are grateful to A. Kondrashev and P. Forterre for fruitful discussions; M. Ares, J. Hughes, P. Mitchel, and N. Grebenshikov for critical reading of and help in preparing the manuscript; and L. Baratova for support.

REFERENCES

- Cline, S. W., and W. F. Doolittle. 1992. Transformation of members of the genus *Haloarcula* with shuttle vectors based on *Halobacterium halobium* and *Haloferax volcanii* plasmid replicons. *J. Bacteriol.* 174:1076-1080.
- Cline, S. W., W. L. Lam, R. L. Charlebois, L. C. Schalkwyk, and W. F. Doolittle. 1989. Transformation methods for halophilic archaeobacteria. *Can. J. Microbiol.* 35:148-152.
- Couturier, M., F. Bex, P. L. Bergquist, and W. K. Maas. 1988. Identification and classification of bacterial plasmids. *Microbiol. Rev.* 52:375-395.
- DasSarma, S., T. Damerval, J. G. Jones, and N. Tandeau de Marsac. 1987. A plasmid-encoded gas vesicle protein gene in a halophilic archaeobacterium. *Mol. Microbiol.* 1:365-370.
- Ebert, K., W. Goebel, and F. Pfeifer. 1984. Homologies between heterogeneous extrachromosomal DNA populations of *Halobacterium halobium* and four new halobacterial isolates. *Mol. Gen. Genet.* 194:91-97.
- Gutierrez, M. C., M. T. Garcia, A. Ventosa, J. J. Nieto, and F. Ruiz-Berraquero. 1986. Occurrence of megaplasmids in halobacteria. *J. Appl. Bacteriol.* 61:67-71.
- Hackett, N. R., and S. DasSarma. 1989. Characterization of the small endogenous plasmid of *Halobacterium* strain SB3 and its use in transformation of *H. halobium*. *Can. J. Microbiol.* 35:86-91.
- Hackett, N. R., M. P. Krebs, S. DasSarma, W. Goebel, U. L. Rajbhandary, and H. G. Khorana. 1990. Nucleotide sequence of a high copy number plasmid from *Halobacterium* strain GRB. *Nucleic Acids Res.* 18:3408.
- Hall, M. J., and N. R. Hackett. 1989. DNA sequence of a small plasmid from *Halobacterium* strain GN101. *Nucleic Acids Res.* 17:10501.
- Hattori, M., and Y. Sakaki. 1986. Dideoxy sequencing method using denatured plasmid templates. *Anal. Biochem.* 152:232-238.
- Holmes, M. L., and M. L. Dyll-Smith. 1990. A plasmid vector with a selectable marker for halophilic archaeobacteria. *J. Bacteriol.* 172:756-761.
- Ilyina, T. V., and E. V. Koonin. 1992. Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eucaryotes and archaeobacteria. *Nucleic Acids Res.* 20:3279-3285.
- Kagramanova, V. K., N. I. Derckacheva, and A. S. Mankin. 1989. Unusual nucleotide sequence heterogeneity of small multicopy pHSB plasmid from *Halobacterium* strain SB3, an archaeobacterium. *Can. J. Microbiol.* 35:160-163.
- Kagramanova, V. K., N. I. Derckacheva, and A. S. Mankin. 1989. The complete nucleotide sequence of the archaeobacterial plasmid pHSB from *Halobacterium* strain SB3. *Nucleic Acids Res.* 16:4158.
- Krebs, M. P., T. Hauss, P. M. Heyn, U. L. Rajbhandary, and H. G. Khorana. 1991. Expression of the bacterioopsin gene in *Halobacterium halobium* using a multicopy plasmid. *Proc. Natl. Acad. Sci. USA* 88:859-863.
- Mount, D. W., and B. Conrad. 1986. Improved programs for DNA and protein sequence analysis on the IBM personal computer and other standard computer systems. *Nucleic Acids Res.* 14:443-454.
- Novick, R. P. 1987. Plasmid incompatibility. *Microbiol. Rev.* 51:381-395.
- Pfeifer, F., U. Blaseio, and P. Ghahraman. 1988. Dynamic plasmid population in *Halobacterium halobium*. *J. Bacteriol.* 170:3718-3724.
- Rosenshine, I., and M. Mevarech. 1989. Isolation and partial

- characterization of plasmids found in three *Halobacterium volcanii* isolates. *Can. J. Microbiol.* **35**:92-95.
20. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 21. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463-5467.
 22. **Sioud, M., G. Baldacci, P. Forterre, and A.-M. de Recondo.** 1988. Novobiocin induces accumulation of a single strand of plasmid pGRB-1 in the archaeobacterium *Halobacterium* GRB. *Nucleic Acids Res.* **16**:7833-7842.
 23. **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.