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Genetic Variability in *Halobacterium halobium*

F. PFEIFER,* G. WEIDINGER, AND W. GOEBEL

Institut für Genetik und Mikrobiologie der Universität Würzburg, D-8700 Würzburg, West Germany

Halobacterium halobium exhibits an extraordinary degree of spontaneous variability. Mutants which are defective in the formation of gas vacuoles (*vac*) arise at a frequency of 10^{-2}. Other easily detectable phenotypes, like the synthesis of bacterioruberin (Rub) or the synthesis of retinal (Ret) and bacterio-opsin (Ops), the two components which form the purple membrane (Pum) of *H. halobium*, are lost at a frequency of about 10^{-4}. With the same frequency a mutant type appears which exhibits an extremely high variability in these phenotypes. With the exception of the *ret* mutants, all spontaneously arising mutants show alterations, i.e., insertions, rearrangements, or deletions, in the plasmid pHH1. It appears that the introduction of one insertion into pHH1 triggers further insertions, which makes the identification of relationships between phenotypic and genotypic alterations rather difficult. From the analysis of a large number of spontaneous *vac* mutants and their vac^+ revertants it can be concluded that the formation of the gas vacuoles is determined or controlled by plasmid genes. No such conclusion is yet possible for the *rub* mutants, although all mutants of this type so far analyzed exhibit a defined insertion. *pum* mutants which have lost the capability of forming bacterio-opsin carry insertions in the plasmid which are distributed over a rather large region of the plasmid. No strains of *H. halobium* could be obtained which had lost plasmid pHH1 completely.

Halobacteria live under conditions of extremely high salinity, but are obligate aerobic heterotrophs. Since the solubility of oxygen in these ecosystems is very low, the gas vesicles found in many halobacteria might serve to keep the cells near the surface. The purple membrane (bacteriorhodopsin) also encountered in several species of *Halobacterium* provides these organisms with a mechanism for utilizing light as a source of energy (8). These bacteria seem to have retained several properties which distinguish them from most procaryotes but are similar to those observed in a few other procaryotes living in extreme environments. These properties include the absence of peptidoglycan cell walls, the occurrence of ether-linked lipids, and characteristic modifications in the base sequence of rRNA's and tRNA's (13). It is an intriguing question whether these so-called archaebacteria may have conserved genetic structures and mechanisms different from those found in the present procaryotes. Little is known about the genetics of halobacteria and other archaebacteria. The separation of large amounts of satellite DNA from the main DNA on the basis of different guanine plus cytosine contents of these two DNAs has been reported for different species of *Halobacterium* (1, 3, 4). At least for *H. halobium* we could demonstrate that the AT-richer satellite DNA is indistinguishable, by its restriction pattern, from a large plasmid, pHH1, isolated from this species. This finding is complemented by the isolation of other plasmids from halobacteria which share closely related sequence homologies when the halobacteria possess similar biochemical and morphological properties, e.g., rod-shaped cells, formation of bacteriorhodopsin, and gas vacuoles.

In this paper we describe the high variability in phenotypic properties of *H. halobium* and the concomitant frequent occurrence of insertions, rearrangements and deletions in the plasmid pHH1. These two features seem to be, at least in part, correlated.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. halobium* NRC817 (Canadian Research Council) was provided by D. Oesterhelt (Max-Planck-Institut für Biochemie). Cells were grown in salt medium (4 M NaCl, 0.12 M $MgSO_4$, 0.03 M KCl, 0.01 M trisodium citrate, 0.5% Casamino Acids, 1% peptone [Oxoid], pH 7.2) with shaking and illumination at 37°C for 7 days.

Source of reagents. All chemicals were obtained from Merck (Darmstadt) or Serva (Heidelberg). Nutrients and agar were obtained from Oxoid (Wesel). The restriction enzymes *Eco*RI and *Pst*I were provided by H. Mayer, Braunschweig; *Hin*dIII was purchased from Biolabs, Beverly, Mass.

Isolation and characterization of plasmid DNA. Plasmid DNA was isolated and characterized by cleavage with restriction enzymes and agarose gel electrophoresis as described in the accompanying paper (6).

Isolation of mutants. All mutants occurred spon-

taneously and were isolated by plating cells on agar plates containing the above-described medium. *vac* mutants appeared as transparent colonies on the plate. Under the light microscope they appeared as black rods, whereas wild-type cells were white. *rub* mutants were easily visible as slightly purple colonies on the agar plates. *pum* mutants were only selected with *rub* mutants as a starting point. They appeared as white colonies on agar plates.

Isolation of purple membrane and ruberin. Cells were grown in 60-ml cultures, harvested in the stationary phase by centrifugation, and suspended in 0.5 ml of DNase solution (0.5 mg/ml). The lysate was dialyzed against 0.05 M Tris-hydrochloride (pH 8.0) for 12 h and layered on top of a 20 to 45% sucrose density gradient. After centrifugation (SW40, 39,000 rpm, 20°C, 6 h) the visible bands were pooled and measured in a spectrophotometer (350 to 700 nm).

RESULTS

Spontaneous mutations in *H. halobium* affecting pigmentation and formation of gas vacuoles. *H. halobium* NRC817 possesses gas vacuoles (Vac) and synthesizes the carotinoid bacterioruberin (Rub) and the chromoprotein bacteriorhodopsin. Bacteriorhodopsin consists of the carotinoid retinal (Ret) and the protein opsin (Ops) and is the main component of the purple membrane (Pum). The loss of each of these phenotypic characters can be easily monitored by the different appearance of the colonies arising from such mutants. Whereas wild-type colonies are pink and opaque, *vac* mutants retain the pink color but become transparent. *rub* mutants are only slightly purple and opaque when they are vac^+ but transparent when they are in addition *vac*. *pum* mutants are more difficult to detect because the color of bacterioruberin will obscure the slight color of the purple membrane. *pum* mutants were therefore selected by starting from *rub* mutants. The differentation of the *pum* mutants into *ret* mutants and *ops* mutants can be performed by observing reconstitution of the purple membrane in the presence of retinal in *ops* but not in *ret* mutants. When *H. halobium* NRC817 was grown for 20 or more generations in basal medium under aerobic conditions, spontaneous *vac* mutant colonies arose at a frequency of 10^{-2}, and *rub* mutant colonies arose at a frequency of 10^{-4}. Starting with a *rub* mutant strain thus obtained, *vac* mutants were again obtained with a frequency of 10^{-2} and *pum* mutants were obtained with a frequency of close to 10^{-4}. Roughly half of the *pum* mutants were *ret*, and half were *ops*. With a similar frequency, *vac rub* double mutants were obtained when the starting culture was *vac*. Transparent and colorless colonies of *vac rub pum* clones were formed at frequencies of 10^{-2} and 10^{-4}, respectively, when cultures of *pum rub* or *vac rub* double mutant strains were plated.

The reversion rate of these spontaneously induced mutations was tested by growing the mutant strains under the above conditions. It was found that most *vac* mutants can revert at a frequency of 10^{-4} or higher. Revertants of *rub* mutants were also obtained at a frequency of $>10^{-4}$, but none of these revertants had wild-type character, producing only low levels of ruberin and having lost their purple membrane. No *pum* revertants were found among ca. 10^5 colonies tested. It should be mentioned, however, that *pum* revertants with a low content of purple membrane may have escaped detection. The data concerning the spontaneous mutations and reversions are summarized in Fig. 1.

Mutation to high frequency of variability. In addition to the mutant colonies described above, "segmented" colonies were observed when a wild-type strain was plated. The frequency with which this type of spontaneous mutation was observed was also in the order of 10^{-4}. When this mutant strain was grown and plated, a large variety of phenotypically different colonies appeared, some of which were analyzed and are listed in Table 1. Some of these clones exhibited again the hypervariability character for the phenotypes expressed by *pum*, *vac*, and *rub*. It thus appears that this character is entirely different from those of the other sponta-

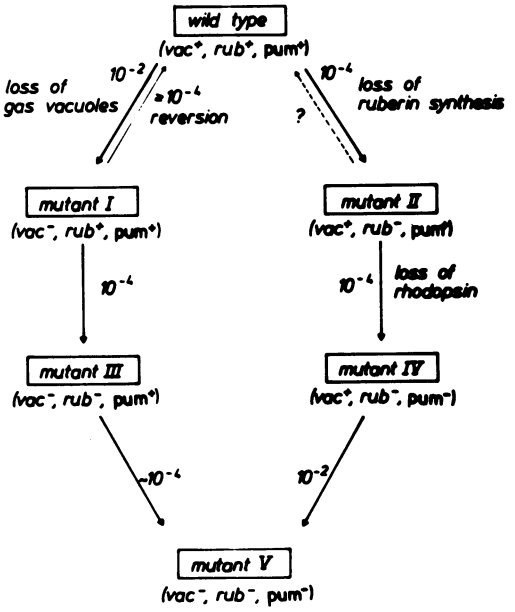

FIG. 1. *Spontaneous mutation rates in H. halobium. The question mark in the reversion of the ruberin loss indicates that the wild-type level of the ruberin synthesis is not restored.*

TABLE 1. *Phenotypic mutants derived from a colony displaying high frequency of variability*

Mutant type	Characters	No. of colonies
rub$^+$ *pum*$^+$ *vac*$^+$	Red, transparent	50
rub pum$^+$ *vac*$^+$	Lycopene accumulation	75
	Purple, opaque	59
	Blue, opaque	64
rub$^+$ *pum vac*$^+$	Orange, opaque	69
rub pum vac$^+$	White, opaque	3
rub pum vac	White, transparent	1
HFV[a] type		8

[a] HFV, High frequency of variability.

neous mutations observed and may affect a gene controlling the mechanism which leads to these spontaneous mutations. We shall call this character high frequency of variability.

Variability in plasmid pHH1. As previously shown (12), *H. halobium* NRC817 carries a 100-megadalton (Mdal) plasmid (pHH1). None of a large number of spontaneously arising mutants tested had lost this plasmid, indicating that plasmid loss was not the cause of their frequent occurrence. To test whether deletions or insertions within the plasmid DNA could lead to the observed phenotypic changes, plasmid DNA was isolated from mutants of each phenotype and cleaved with the restriction endonucleases *Hin*dIII, *Pst*I, and *Eco*RI. The restriction patterns were then compared to that of the plasmid from the wild-type strain. *Hin*dIII cleaves pHH1 into 11 fragments which have been ordered in a physical map as recently described (G. Weidinger, F. Pfeifer, and W. Goebel, Methods Enzymol., in press). As shown in Fig. 2, the two largest *Hin*dIII fragments, H1 and H2, can also be subfragmented by *Pst*I and *Eco*RI, thus also allowing the detection of small changes within these regions of pHH1. With the possible exception of the relatively large region of pHH1 covered by H3, which lacks sites for *Pst*I and carries only a few sites for *Eco*RI, even minor changes in pHH1 should be detected when the plasmid DNA is cleaved with the three restriction enzymes. The known location of each of these restriction fragments on the physical map of pHH1 then allows a fairly precise determination of possible alterations in the plasmid DNA of the mutants. With this experimental approach we made the following observations. (i) With the exception of *ret* mutants, all phenotypically altered mutants exhibited changes in plasmid pHH1 (Fig. 3). (ii) Most of the observed changes represented insertions of various size classes. The most frequently occurring insertion was about 0.35 Mdal, but larger insertions of about 0.4, 0.7, 0.9, 1.0, 1.2, 1.5, and 2.1 Mdal were also observed. (iii) Plasmid DNAs of many mutants contained more than one insertion. It appears that a plasmid which already carries an insertion is more sensitive to the introduction of further insertions, since upon further cultivation of strains harboring a plasmid with one insertion, subclones with plasmids carrying more than one were often found. (iv) Plasmids carrying several insertions were relatively sensitive to deletions within the region where the initial insertion(s) was located (Fig. 2). (v) The occurrence of these insertions in the plasmid does not seem to be a normal event in the wild type, since plasmid DNA from 10 randomly selected wild-type clones (i.e., *pum*$^+$ *vac*$^+$ *rub*$^+$) did not show a single alteration in pHH1. (vi) Revertants selected for the rgeneration of specific phenotypes (see below) showed either an apparently exact removal of a certain insertion or more or less extended deletions in the plasmid pHH1, or both. A summary of these results is given in Fig. 2.

Are phenotypic alterations correlated with alterations in plasmid pHH1? The frequent occurrence of insertions and deletions in *vac*, *pum*, and *rub* mutants prompted us to analyze whether these genes are located on plasmid pHH1. Plasmid DNA from several independently islated *vac* mutants, i.e., I-1, I-4, I-5, II-4, II-5, II-6, III-1, III-2, III-3, and V-1 (Fig. 2a) showed common insertions. Most *vac* mutants carried an insertion of 0.4 Mdal which was located on the *Pst*I fragment P9. In all *vac* mutants another insertion was found in the *Eco*RI fragment E6. All insertions in P9 seemed to be at the same position, although we do not know whether changes in the orientation may occur. In E6, at least two different sites have been recognized by cleaving E6 with *Hin*dIII, where the insertion may occur. In most *vac* mutants the insertion (either 0.35 or 1.2 Mdal) was located on the H2 part, but in the two mutants III-2 and III-3 the insertion (0.7 Mdal) was on the H3 part of E6. In the *vac* mutant V-1, a large region of pHH1 including E6 was deleted. All additional insertions that occurred in the various *vac* mutants did not seem to be connected with gas vacuole formation (see below). It seems to be rather unlikely that the insertion (0.4 Mdal) in P9 is connected with the loss of gas vacuoles, since mutants II-1, II-2, II-3, IV-1, and IV-2, which are *vac*$^+$, seemed to have the same insertion. On the other hand, the *vac* mutants II-4, II-5, and II-6 do not carry this insertion. The insertion(s) in E6 is most likely linked to the loss of gas vacuole formation, since in most *vac*$^+$ revertants, this insertion was lost. The removal of this insertion was, in many *vac*$^+$ revertants, accompanied by the concomitant loss of other

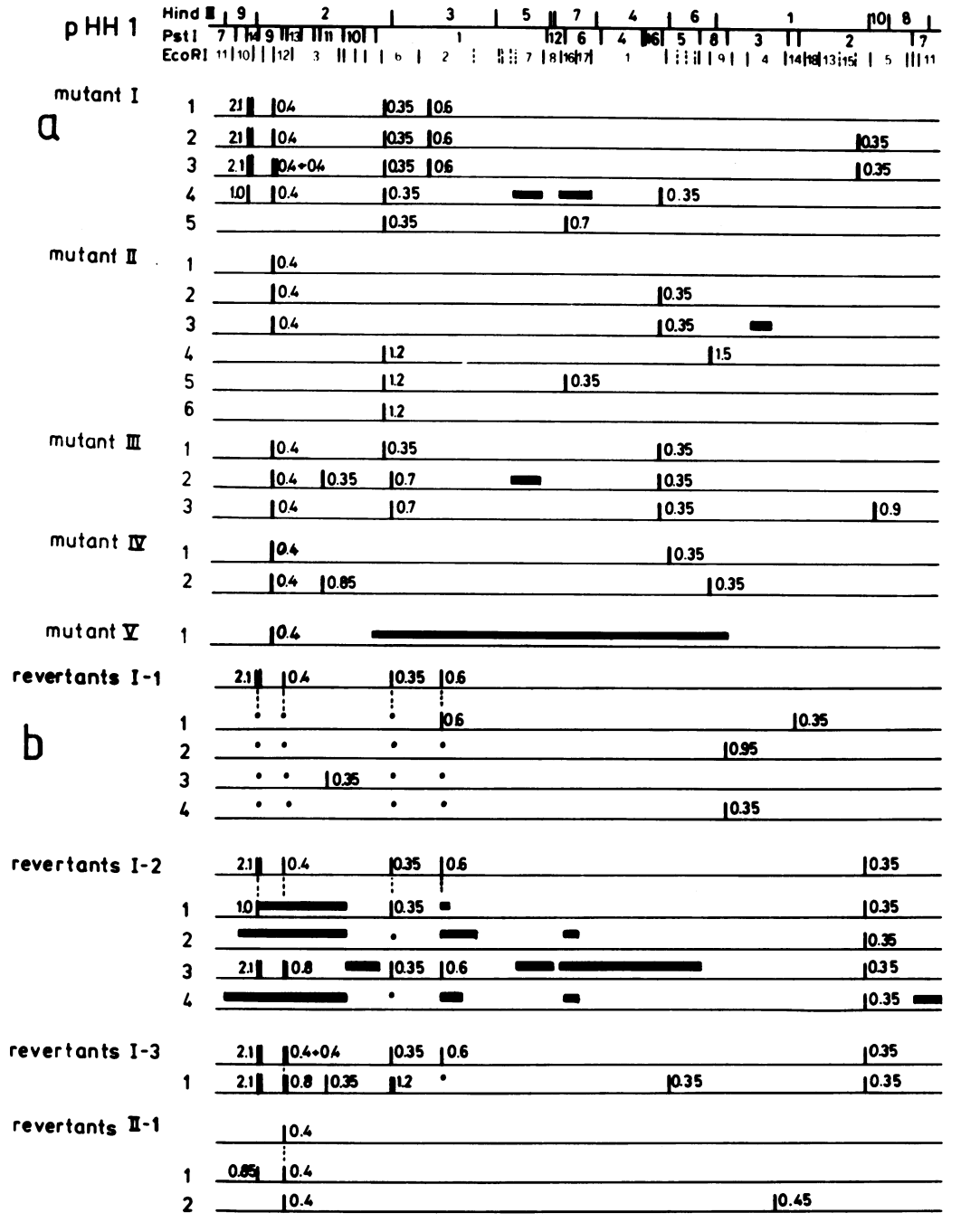

FIG. 2. *Restriction map of pHH1 in comparison with several mutants and revertants. (a) Most of the mutants are independently isolated with these exceptions: mutants I-2 and I-3 derive from I-1, mutants II-2, II-3, and IV-1 derive from rub mutant II-1, and mutant III-1 was from II-2. The mutants II-4, II-5, and II-6 accumulate lycopene but do not synthesize bacterioruberin. (b) The first three groups are revertants of mutant I-1, I-2, or I-3, i.e., reversion of gas vacuole formation. Revertants of rub mutant II-1 have restored ruberin synthesis but have never reached wild-type level. Numbers in the restriction maps indicate insertion size in Mdal; thick black lines show deletions; a black point represents reversion of the insertion.*

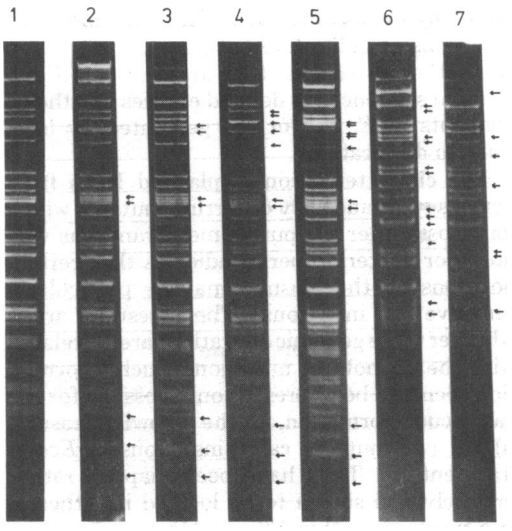

FIG. 3. *EcoRI restriction pattern of the wild-type plasmid pHH1 (lane 1) and of several mutants: (2) rub mutant II-2; (3) to (5) the vac mutants III-1, III-3, and III-2, respectively; (6) vac mutant I-2; and (7) revertant II-23. Arrows indicate alterations in the restriction patterns.*

insertions or by deletions. The revertants I-11 to I-14 (Fig. 2b), all of which derive from the *vac* mutant I-1, may lose three or four insertions including that in E6 at the same time, but may gain insertions of different sizes in other parts of pHH1. Two of four *vac*$^+$ revertants deriving from the *vac* mutant I-2 also lost the insertion in E6. Two others, I-21 and I-23 (the latter of which is only partially *vac*$^+$), still retained the 0.35-Mdal insertion in E6 but suffered extended deletions close to the site of this insertion. In one *vac* mutant, I-3, an insertion of 1.2 Mdal was observed in the *Eco*RI fragment E6. This could indicate that this revertant retained the 0.35-Mdal insertion in E6 but gained in addition another insertion of 0.85 Mdal located close to the previous one, or that a new insertion of 1.2 Mdal with different properties is present in E6 (Fig. 2).

All *rub* mutants that occurred spontaneously carried plasmid pHH1 with an insertion of 0.4 Mdal in the *Pst*I fragment P9 or in the *Eco*RI fragment E12 (Fig. 3). The mutants II-4 to II-6 (Fig. 2a), which lack this insertion but are unable to synthesize ruberin, are different from those described earlier in that they accumulate large quantities of lycopene. Since lycopene, regarded as a precursor of ruberin, is not accumulated in *rub* mutants carrying the 0.4-Mdal insertion in P9, one could argue that this insertion affects an early step in ruberin synthesis. However, there are several lines of evidence which do not support this interpretation. (i) The mutants I-1 to I-4 are *vac* but *rub*$^+$, yet they also carry this insertion. It should be mentioned, however, that the orientation of the 0.4-Mdal insertion in P9 is unknown and could well be different in these mutants from that in the *rub* mutants. Besides, all four mutant plasmids contain, close to this insertion, one or two additional insertions of about 1.0 Mdal (Fig. 2a), which could overcome the effect of the first one. (ii) *vac*$^+$ revertants (I-21, I-22, and I-24) have been found which carry deletions in P9 and the neighboring region of pHH1 (Fig. 2b) and are still *rub*$^+$. (iii) The strains *H. trapanicum* and *H. volcanii* and a halophilic isolate from Israel are able to form a red pigment which is most likely ruberin (6), but neither the chromosomal DNA nor plasmid DNA, if present, isolated from these strains hybridizes with pHH1 sequences.

rub$^+$ revertants (II-11 and II-12; Fig. 2) were obtained by starting from the *rub* mutant II-1, which contains only the 0.4-Mdal insertion in P9. These revertant clones could synthesize ruberin only in low quantities and lost concomitantly the capability of purple membrane synthesis. The P9 insertion in plasmid pHH1 was not eliminated in these revertants, but in each of them a new insertion appeared (Fig. 2b).

There are two types of purple membrane (*pum*) mutants, both of which were found to arise spontaneously. Type I lacks bacterio-opsin (*ops*), and type II fails to synthesize retinal (*ret*). Purple membrane synthesis can be reconstituted in type II *pum* mutants by the addition of retinal to the growth medium (5, 10). No changes have been observed in the plasmid pHH1 isolated from several *ret* mutants, which indicates that pHH1 most likely does not determine functions required for retinal synthesis. In contrast, all *ops* mutants show more or less extensive changes in pHH1. Whereas mutant V (Fig. 2) has suffered an extended deletion which affected half of the DNA molecule, other *ops* mutants carry one or more insertions, like mutants IV-1 and IV-2.

These insertions are in other mutants, however, widely distributed from P6 to P2 on the physical map of pHH1 (data not shown). Most of these *ops* mutants arise from a *vac*$^+$ *pum*$^+$ *rub* *H. halobium* strain, which carries a plasmid with sequences arranged differently from those of plasmid pHH1, for which the physical map has been constructed. This makes conclusions concerning a possible correlation between the *ops* mutations and changes within plasmid pHH1 rather difficult at the moment.

The mutation leading to the high frequency of variability character, i.e., high frequency of variability with regard to the three genotypes *vac*, *pum*, and *rub*, does not seem to affect the

plasmid DNA. All differently colored *H. halobium* clones deriving from this segmented mutant seemed to harbor plasmids with no apparent change in pHH1 compared to the parental plasmid. Since all of these clones are altered in carotinoid biosynthesis, this further suggests that genes controlling carotinoid biosynthesis are not determined by the plasmid. Only *vac* mutants that derived from a high frequency of variability mutant contained insertions in E6, which further supports the conclusion that pHH1 carries genes for gas vacuole formation.

DISCUSSION

As shown in the previous publication (6), most of the extremely halophilic bacteria share common DNA sequences which are found in some strains on covalently closed circular DNAs (plasmids) and in others possibly on the chromosome. These sequences are not shared by other only moderately halophilic species, regardless of whether or not they carry plasmids. As shown for *H. halobium* (9), these sequences are indistinguishable from the minor satellite DNA component which possesses a lower guanine plus cytosine content (57 to 60 mol%) than the chromosome (66 to 68 mol%) and has been identified in this and other species of extremely halophilic halobacteria and halococci (3). In addition to their different halophilic properties, halobacteria of the first group often form gas vacuoles, bacteriorhodopsin, and bacterioruberin(s), which the others (with the possible exception of bacterioruberin) fail to synthesize. It is shown in this paper and in previous ones (2, 9, 12) that these genetic characters are lost in *H. halobium* at frequencies which are extremely high for chromosomally inherited functions. However, this event is not accompanied by the loss of a plasmid, as already indicated by the observation that some spontaneously arising *vac* or *rub* mutants can revert. A possible mechanism for the generation of the spontaneous mutations is the appearance of insertions which seem to occur in *H. halobium* in most of the phenotypic mutants. We have reported here only on the occurrence of insertions on plasmid pHH1 since they can be more easily detected by restriction analysis and mapped on this extrachromosomal element. Howevr, there is circumstantial evidence that such insertions do also occur within the main band of *H. halobium* DNA (Pfeifer, unpublished data). If insertions are the major cause for the genetic variability in halobacteria, it can be expected that chromosomal determinants will be affected with a similar frequency. This assumption is only justified if the insertions observed here behave like the IS elements known from *Escherichia coli* (7). However, we do not as yet have any evidence as to whether *H. halobium* insertions of quite different sizes are related to each other or whether they are transposed into random sequences as defined entities like the IS elements in *E. coli* or are generated by local tandem duplications.

The characterization of plasmid DNA from various spontaneously occurring mutants which have lost either the purple membrane, gas vacuoles, or bacterioruberin indicates that certain positions on the plasmid may be particularly sensitive to insertions. The question arises whether these genomic alterations are correlated with the phenotypic mutations. Such a correlation seems to be at present only possible for the gas vacuole formation, for the following reasons. (i) All *vac* mutants carry insertions in *Eco*RI fragment E6. They have been mapped rather precisely and shown to be located in either of two positions within E6. (ii) Most vac^+ revertants have lost these insertions, whereas a few others carry a second insertion or deletion very close to the first insertion. This second event may overcome the effect of the first insertion, if for example the first insertion inactivates a promoter which controls the transcription of the genes determining the gas vacuole proteins (11) and the second insertion carries a promoter or the deletion brings another plasmid promoter close to these genes. Such conclusions between genomic and phenotypic alterations are hampered, however, by the frequent occurrence of additional insertions which are apparently unrelated to a given phenotype and by the concomitant disappearance of more than one insertion in revertants. There are, for example, vac^+ revertants which have lost at the same time insertions of different sizes in four rather remote positions of the plasmid, three of which are most likely unrelated to the gas vacuole formation. This problem has led us before to the incorrect conclusion that gas vacuole formation is linked to an insertion in *Pst*I fragment P14 (12).

Although the 0.4-Mdal insertion in *Pst*I fragment P9 was observed in all *rub* mutants, there is experimental evidence which indicates that this locus is not directly linked to bacterioruberin synthesis. This further suggests that there may be insertion "hot spots" in the plasmid. Another position of this type seems to be the *Eco*RI fragment E23, where a 0.35-Mdal insertion has been frequently observed. It remains an open question why all plasmids from *rub* mutants show insertions or other alterations whereas none of the plasmid DNAs from *ret* mutants is changed, if neither of the two carotinoids is controlled by plasmid genes. The other type of bacteriorhodopsin mutant which is unable to form opsin again shows alterations

within the plasmid. These insertions or deletions are, however, spread over a rather large region of the plasmid. This could again suggest that the changes are unrelated to the phenotype, or it could indicate that a large number of genes are required for the formation and modification of opsin, making it suitable for the formation of purple membrane in the presence of retinal. All of these open questions can only be answered when we know more about the mechanism by which the insertions occur and the possible interactions between plasmid and chromosome.

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