JOURNAL OF BACTERIOLOGY, Apr. 1985, p. 461–462
0021-9193/85/040461-02$02.00/0

Vol. 162, No. 1

Genetic Transfer in *Halobacterium volcanii*

MOSHE MEVARECH* AND RUTH WERCZBERGER

Department of Microbiology, George S. Wise Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv, Israel

Received 10 September 1984/Accepted 10 January 1985

Auxotrophic mutants of *Halobacterium volcanii* generated by chemical mutagenesis were used to demonstrate a native genetic transfer system in this extremely halophilic member of the class *Archaeobacteria*.

Bacteria of the genus *Halobacterium* are obligatory halophiles that survive only in extremely high salt concentrations by accumulating even higher intracellular amounts of salts (3). Although the biochemistry of these microorganisms has been extensively studied (1), their genetics is still unexplored. Genetic studies are difficult to perform because of the fact that most of the studied halobacteria are chemoorganotrophic, requiring a complex nutrient medium for growth. Moreover, halobacteria are highly resistant to most drugs that are used to screen for genetic markers (2). Recent studies have shown that some species can use simple organic molecules as their sole carbon source (5). In this report, one of these species was used to obtain auxotrophic mutants and for subsequent genetic studies.

Halobacterium volcanii DS2 (4), obtained from Moshe Shilo (Hebrew University, Jerusalem, Israel), was used in this study. Bacteria were grown in a salt mix containing (per liter) 206 g of NaCl, 36.9 g of $MgSO_4 \cdot 7H_2O$, 5 ml of 1 M KCl, and 1.7 ml of a 75-mg/liter concentration of $MnCl_2$. When minimal medium was prepared, the following components were added: 5 ml of 1 M NH_4Cl, 45 ml of 10% glycerol, 5 ml of 10% sodium succinate, 1 ml of trace elements (30 mg of $MnCl_2$, 44 mg of $ZnSO_4$, 230 mg of $FeSO_4$, and 5 mg of $CuSO_4$ per 100 ml), and 2 ml of 0.5 M K_2HPO_4. For complete medium, 5 g of yeast extract (Difco Laboratories, Detroit, Mich.) per liter was added. In each case, after the medium was autoclaved and cooled, 5 ml of 10% $CaCl_2 \cdot 2H_2O$ was added, and the pH was adjusted to 7.2. When needed, amino acids (final concentration, 50 μg/ml) or adenine (final concentration, 25 μg/ml) was added to minimal medium. For agar plates, 25 g of Bacto-Agar (Difco) per liter was added. Bacteria were grown at 42°C with shaking.

Mutagenesis was performed in salt mix containing 0.2 M Tris-hydrochloride (pH 7.5). To 6 ml of the bacterial suspension, 90 μg of ethyl methanesulfonate (Eastman Kodak Co., Rochester, N.Y.) was added and dissolved by vigorous vortexing. The culture was shaken for 2 h at 37°C. The cells were then collected by centrifugation, washed, and injected into complete medium. When the cell density reached 10^9 cells per ml, the cell suspension was diluted and plated on agar plates of complete medium. The colonies obtained were screened for auxotrophy. Those colonies growing on complete medium but not minimal medium were transferred to a set of plates containing various mixtures of amino acids, purines, and pyrimidines to identify the nutritional requirements. Once the nutritional requirements were determined, they were verified by plating the colonies on agar plates of minimal medium supplemented with the required nutrient. Of 2,250 colonies tested, 50 did not grow on minimal medium, and the requirements of 23 were identified. Fourteen of the mutants were adenine auxotrophs. Table 1 shows a list of all the available mutants.

To test whether genetic transfer occurred, we grew each of the auxotrophic mutants WR101 (Ade), WR205 (Pro), and WR206 (Phe) in a liquid culture inoculated with a single colony. Paired combinations of the mutants were mixed in the desired proportions (in most cases, 1:1), and then each culture was filtered through a nitrocellulose filter (BA85, 25-mm diameter; Schleicher & Schuell Inc.). The filters were placed on agar plates of complete medium and incubated for 96 h. During this time, a red mat of bacteria appeared. The filters were removed, and the bacteria were suspended by being shaken in salt mix for 30 min at room temperature. The cells were washed, suspended in salt mix, diluted, and plated on agar plates of either minimal medium or complete medium. The plates were incubated at 42°C. Colonies appeared after 6 days on agar plates of complete medium and after 10 days on agar plates of minimal medium. The putative prototrophs obtained were confirmed to be real prototrophs and not the result of cross-feeding between the two parental strains. Individual prototrophic colonies were diluted and spread on agar plates of either complete medium or minimal medium. The number of colonies that grew on both media were similar. In each experiment, a control for spontaneous reversion was used. The results are shown in Table 2.

To determine whether prolonged contact between the cells was essential for genetic transfer, we grew two auxotrophic strains as a shake culture in complete medium or allowed them to coprecipitate under gravitational force at 37°C for 48 h. No transfer was detected when the mixed culture was constantly agitated. On the other hand, 40 colonies out of 5.8×10^9 cells that coprecipitated were

TABLE 1. Available auxotrophic mutants of *H. volcanii*

Strain(s)	Phenotype	Requirement
WR101 through WR114	Ade	Adenine
WR201	His	Histidine
WR202	Lys	Lysine
WR203	Ser	Serine
WR204	Gly	Glycine
WR205	Pro	Proline
WR206 and WR207	Phe	Phenylalanine
WR208	Cys	Cysteine
WR209	Gua	Guanine

* Corresponding author.

prototrophs. The addition of DNase at a concentration of 100 μg/ml to either the mixed culture on the mat or to the coprecipitated cells increased the number of prototrophs 6 and 30-fold, respectively. Although it is not clear why the DNase treatment facilitated the transfer, it is clear that the genetic transfer was not a simple DNase-sensitive transformation. No spontaneous revertants were observed in these experiments.

The possible participation of a bacteriophage in the transfer was checked by filtering a liquid culture of one mutant through a 0.22-μm-pore-size nitrocellulose filter and layering the filtrate on the cell mat of the other mutant. No prototrophs were obtained, suggesting that the transfer does not occur by transduction.

Indirect evidence for active genetic transfer was obtained from an experiment in which heat-killed cells (70°C, 90 min) of one mutant were cofiltered through a nitrocellulose filter with cells of the other mutant. Under these conditions, less than 10^{-4} heat-treated cells survived. No transfer was detected. Because genetic transfer was observed between any two of the three mutant phenotypes (Ade, Pro, or Phe) that were checked, we can argue that the transfer is not unidirectional, as is the case in classical conjugation known in other procaryotes.

The genetic transfer was used to distinguish between different mutations affecting the same phenotype. The test was performed by means of crosses between the 14 adenine auxotrophs. The results are shown in Table 3. It can be seen that the mutants fall into at least three groups: (i) WR101, WR104, WR105, WR106, WR108, WR109, WR110, WR111, and WR113; (ii) WR102, WR107, and WR114; and (iii) WR103 and WR112. No spontaneous revertants were observed in these experiments.

TABLE 2. Results of crosses between Ade, Pro, and Phe auxotrophs

Phenotype of bacteria on mat	No. of cells plated on selective plates	No. of prototrophs	Frequency of transfer
Ade	6.5×10^8	0	
Pro	1.2×10^9	0	
Phe	1.0×10^9	0	
Ade×Pro	1.3×10^9	2.9×10^3	2.2×10^{-6}
Ade×Phe	1.3×10^9	4.1×10^3	3.1×10^{-6}
Pro×Phe	1.3×10^9	3.8×10^3	2.9×10^{-6}

TABLE 3. Results of crosses between different Ade auxotrophs[a]

Cross	No. of cells plated on selective plates	No. of prototrophs
WR101 × WR102	4.1×10^8	8.8×10^3
WR101 × WR103	2.8×10^8	2.3×10^3
WR101 × WR107	3.6×10^8	8.0×10^3
WR101 × WR112	2.0×10^9	7.0×10^2
WR101 × WR114	3.2×10^9	2.6×10^4
WR102 × WR103	6.2×10^9	5.0×10^2
WR102 × WR112	2.9×10^9	2.0×10^4

[a] No prototrophs were obtained with the following crosses: WR101 × WR104, WR101 × WR105, WR101 × WR106, WR101 × WR108, WR101 × WR109, WR101 × WR110, WR101 × WR111, WR101 × WR113, WR102 × WR107, WR102 × WR114, WR103 × WR112.

At the moment, the system described enables a distinction to be made between different mutations affecting the same phenotype. To be able to use this system for genetic mapping, we will have to isolate many more mutants and double mutants and determine the average amount of genetic information transferred at each event. As this is the first genetic transfer system reported so far in *Archaebacteria*, it will be interesting to see whether similar systems exist in other genera of this class.

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