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Selectable Mutant Phenotypes of the Extremely Thermophilic Archaebacterium *Sulfolobus acidocaldarius*

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As a first step toward developing the genetic potential of extremely thermophilic archaebacteria, mutant strains of *Sulfolobus acidocaldarius* were selected by plating cells directly on solid medium containing one of several growth inhibitors. Three spontaneous resistance phenotypes were observed (5-fluorouracil resistance, novobiocin resistance, and L-ethionine resistance), each at a different average frequency. Characterization of representative strains showed each of the three mutant phenotypes to provide a potentially useful genetic marker.

Efforts to understand organisms classified as Archaea (12), more commonly known as archaebacteria, at the molecular level have, to date, been dominated by two approaches: (i) classical biochemistry, including the purification and characterization of individual enzymes and low-molecular-weight compounds and (ii) molecular cloning, typically used to establish the evolutionary relatedness of macromolecules via sequence analysis. As a result, unique molecular aspects of archaebacteria have been discovered whose functional significance in vivo can only be speculated. This disparity of knowledge is particularly acute for the Crenarchaeota (12), sulfur-metabolizing, extremely thermophilic archaebacteria found in geothermal habitats, whose unusual physiology is accompanied by enzymatic activities (6), lipid structures (9), and low-molecular-weight compounds (8) that appear novel even by archaebacterial standards.

Establishing the functional significance of novel features of extremely thermophilic archaebacteria will generally require the application of basic genetic strategies to one or more of these organisms. Several groups have for several years concentrated on applying genetic techniques to halophilic or methanogenic archaebacteria, with reasonable success (2, 5, 7, 10). Furthermore, this researcher has provided evidence that with respect to a combination of basic qualifications for genetic manipulation (colony formation on solid medium, growth on simple compounds as the sole carbon or nitrogen source, metabolic and nutritional versatility, tolerance of various culture conditions, amenability to routine biochemical analysis, and sensitivity to certain antibiotics and metabolite analogs), strains of the genus *Sulfolobus* compare favorably with any other archaebacterium (4). However, development of genetic techniques for *Sulfolobus* strains, including methods of gene transfer, has to date been precluded by the lack of selectable genetic markers. Here I demonstrate the straightforward isolation of mutant strains of *Sulfolobus acidocaldarius* which appear useful for genetic experimentation.

Growth conditions were essentially those described previously (4); all media contained 0.2% carbohydrate (starch, L-arabinose, or D-xylose) as the primary carbon source and 0.1% amino acid (gelatin, peptone, or L-glutamine) as the nitrogen source and were adjusted to a pH of 3.4 to 3.8 at room temperature before inoculation. Strain DG6 was derived from *S. acidocaldarius* strain C (4), by two cycles of the following procedure: (i) cells were spread on solid medium to yield confluent growth, (ii) papillae were streaked for isolation, and (iii) the largest of the resulting isolated colonies was taken. Strain DG6 retained the prototrophic, pentose-utilizing phenotype of strain C (4) and was used as the parental (wild-type) strain except where otherwise noted; it has been deposited with the American Type Culture Collection (ATCC 49426).

To select resistant mutants, cultures were grown from mass inocula (containing about 10^7 cells) to late exponential or early stationary phase. Up to 5×10^8 cells were plated directly on solid medium containing 1 of 10 inhibitors, identified in a previous study (4), at approximately twice the MIC. In three cases (described below), after 5 to 8 days of incubation, isolated colonies formed from which viable cells were recovered. Further incubation yielded no additional colonies. Representative colonies were streaked for isolation before being further characterized. All three resistance phenotypes appeared stable, in that all isolated colonies grown on nonselective plates scored resistant upon subsequent challenge with selective agent. In some cases, resistant strains were also propagated for several generations in nonselective liquid medium, with no resulting decrease in the observed level of resistance (MIC).

Fluorouracil resistance. The best characterized of the mutant phenotypes was resistance to 5-fluorouracil (Fur^r). On various media containing 60 to 200 μg of 5-fluorouracil per ml, Fur^r colonies arose at frequencies of 2×10^{-7} to 1×10^{-6} per cell plated. For each of seven isolates tested, the acquired resistance was well above that required to grow in the original selective medium; this property is illustrated by the independent mutants DG14 and DG35 (Table 1). The biochemical basis for this phenotype was not determined. Analogous phenotypes have been observed in other organisms, however, including methanogenic archaebacteria (1, 7, 10), and are generally due to loss of nonessential pyrimidine salvage activities (1, 10). The relatively high spontaneous frequency of the Fur^r phenotype in *S. acidocaldarius*, as

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TABLE 1. Summary of mutant phenotypes

Selective agent[a]	Designation of representative strain	Observed MIC[b] (μg/ml) of drug		
		5-Fluorouracil	Novobiocin[c]	L-Ethionine
None	DG6	40, 60, <30	3, 5 (4)	<60, <60
Novobiocin (4)	DG8[d]	ND	10 (6)	ND
L-Ethionine (400)	DG12	<30	3	>2,000, >4,000
5-Fluorouracil (200)	DG14	>2,000	ND	<60
5-Fluorouracil (160)	DG35[e]	>2,000	ND	>4,000

[a] Numbers in parentheses indicate the dose (microgram of selective agent per ml of solid medium).

[b] Unless otherwise noted, determined by serial 1:2 dilution in liquid medium as previously described (4); inoculum size was approximately 3×10^6 cells, and growth was scored after 4 days of incubation. Multiple values reflect indpendent trials, in which the combination of carbon and nitrogen sources were varied (see text). ND, not determined.

[c] Values in parentheses were determined by scoring growth on a series of plates containing 0, 4, 6, 8, or 10 μg of novobiocin per ml.

[d] Derived directly from strain C, rather than from strain DG6.

[e] Derived from a strain, DG13, identical to DG12 in derivation and phenotype.

TABLE 2. Estimation of mutation frequencies

Trial no.[a]	Strain(s) plated	No. of cells[b]		No. of colonies obtained	Apparent frequency
		Sensitive	Resistant		
1	DG6	1.5×10^8		0	$<6 \times 10^{-9}$
	DG6 + DG12	1.5×10^8	330	244	
2	DG6	6×10^8		1	1×10^{-9}–2×10^{-9}
	DG6 + DG12	8×10^7	500	392	
	DG6 + DG12	8×10^7	1,000	731	
	DG6 + DG12	8×10^7	2,000	1,644	
3	DG6	1×10^8		39	4×10^{-7}
	DG6 + DG14	1×10^8	550[c]	417	
	DG6 (UV)[d]	1×10^8		980	1×10^{-5}
	DG6 (Et_2SO_4)[d]	1×10^8		1,290	1×10^{-5}

[a] The following media were used (concentration of selective agent given in parentheses [micrograms per milliliter]; see text for details): trail 1, xylose-glutamine plus L-ethionine (150); trial 2, xylose-peptone plus L-ethionine (150); trial 3, xylose-peptone plus 5-fluorouracil (160).

[b] Calculated on the basis of culture turbidity, assuming 1.0×10^9 cells per ml per A_{600} under the conditions employed (estimated in earlier studies by microscopic count of strain C).

[c] Includes 40 CFU from line above.

[d] Cultures treated with the indicated mutagen as described in text. Et_2SO_4, diethyl sulfate.

well as the gratuitous levels of resistance obtained (Tables 1 and 2) seem consistent with such a mechanism of resistance.

Novobiocin resistance. In its original description, *S. acidocaldarius* was reported to be novobiocin sensitive (3), and this antibiotic is among the most effective of those shown to inhibit growth of *Sulfolobus* strains (4). Mutants resistant to 4 μg of novobiocin per ml of solid medium arose at an apparent frequency of 10^{-6} to 10^{-5} per cell plated. The representative Nov^r isolates from one such plating showed a rather modest increase in resistance compared to that of the wild type (Table 1). Successive transfer of these strains to increasingly higher concentrations in liquid medium resulted in apparent habituation to novobiocin concentrations as high as 16 μg/ml, but after a few generations growth in nonselective liquid medium, plating up to 10^8 of these cells on solid medium containing 6 μg of novobiocin per ml yielded neither confluent growth nor isolated colonies. Thus, the experience of Holmes and Dyall-Smith (5) with *Haloferax* strains, in which 1,000-fold resistance to novobiocin was obtained by a similar regimen, was not observed for *S. acidocaldarius*. It may be relevant that wild-type *Sulfolobus* strains tolerate about 400 times the concentration of novobiocin tolerated by the wild-type *Haloferax* strain (4, 5). In any case, the relatively small difference between drug MICs for wild-type and mutant strains did not preclude use of the Nov^r phenotype as a nonselected genetic marker. The discriminating thresholds were found to be well-defined and reproducible, so that strains could be readily scored on selective medium by standard techniques of replica plating, spotting, etc. (data not shown).

Ethionine resistance. The third mutant phenotype observed in this study was resistance to L-ethionine (Eth^r). As did Fur^r strains, Eth^r mutants proved resistant to concentrations of selective agent far above those used in their isolation (Table 1); unlike the previous two cases, however, the apparent frequency of mutation was quite low, i.e., about 2×10^{-9} per cell plated. This latter observation, which included failure to recover any Eth^r colonies in several trials, could have resulted from a low efficiency of plating overall, caused by the use of relatively poor carbon and nitrogen sources in the selective medium; such an effect has been reported for another *Sulfolobus* species (11). Mutant isolation experiments were therefore repeated, using lower concentrations of L-ethionine, different medium formulations, and the addition of small numbers of DG12 cells to the plating mixture as an internal control for the plating efficiency of Eth^r cells. The results (Table 2) show that under the conditions used to select spontaneous Eth^r mutants (i.e. at least 5×10^7 sensitive cells per plate), Eth^r cells formed colonies at greater than about 70% efficiency. The low frequency at which Eth^r mutants were recovered thus seems due to an inherently low spontaneous frequency for this phenotype.

Finally, to test the effect of mutagenic treatments on the apparent frequency of resistance mutations, about 5×10^7 DG6 cells were incubated with sufficient diethyl sulfate (0.1%) to slow growth considerably, and about 3×10^8 DG6 cells were irradiated to about 1% survival (6×10^7 erg/cm^2) with a germicidal UV lamp under normal laboratory lighting. In both cases, the resulting cells were washed and grown for at least 5 to 6 generations in fresh medium. As shown in Table 2, recovery of the Fur^r phenotype increased 25- to 30-fold after exposure to UV or chemical mutagen, providing additional evidence that the Fur^r phenotype results from simple genetic mutation. A preliminary experiment in which Nov^r was selected yielded qualitatively similar results (not shown), although the apparent frequencies were correspondingly higher.

These results document the first isolation of *Sulfolobus* mutants exhibiting well-defined growth phenotypes and demonstrate that certain standard techniques of bacterial genetics can be applied to archaebacteria from geothermal habitats. The three mutant phenotypes characterized in this study represent easily scored genetic markers which can be incorporated in a straightforward manner into *S. acidocaldarius* and presumably into other *Sulfolobus* strains as well. They can also be combined in a given strain by sequential selection, as illustrated by construction of the doubly mutant strain DG35 (Table 1).

One obvious use for a selectable marker in *Sulfolobus* strains is the detection of genetic exchange, which is crucial to the development of transformation and other modern

genetic techniques for extremely thermophilic archaebacteria. Although their high spontaneous frequencies make the Nov^r and Fur^r phenotypes unsuitable for this purpose, the relatively rare Eth^r phenotype may prove effective as a selected marker, as may additional resistance mutations isolated with the help of mutagenesis. Also, the relatively frequent Nov^r and Fur^r phenotypes seem to provide convenient measures of spontaneous mutation in *S. acidocaldarius*. This may open certain fundamental questions to laboratory experimentation, including whether environmental factors limit the accuracy of DNA replication in organisms populating geothermal habitats, as well as the possible significance of spontaneous degradation of DNA for the survival and evolution of archaebacteria in geothermal habitats.

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