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Sulfolobus acidocaldarius Synthesizes UMP via a Standard De Novo Pathway: Results of a Biochemical-Genetic Study

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A genetic approach was used to establish the route of UMP biosynthesis in *Sulfolobus acidocaldarius*, a member of the hyperthermophilic division (the *Crenarchaeota*) of the *Archaea* domain. Pyrimidine auxotrophs of *S. acidocaldarius* DG6 were isolated by direct selection and by brute-force methods. Enzymatic assay of extracts from wild-type *S. acidocaldarius*, from pyrimidine auxotrophs, and from phenotypic revertants demonstrated that *S. acidocaldarius* synthesizes UMP via orotate in six enzymatic steps corresponding to the de novo pathway of other organisms. The results also show that a single carbamoyl phosphate synthetase supplies both the pyrimidine and arginine pathways of this organism. To gain similar insight into pyrimidine salvage pathway(s), prototrophic mutants resistant to toxic pyrimidine analogs were also isolated and characterized. The results suggest that a single class of mutants which had acquired elevated resistance to four different 5-fluoropyrimidines had been isolated. These fluoropyrimidine-resistant mutants appear to have a regulatory defect leading to overproduction of one or more endogenous pyrimidine compounds.

Members of the *Archaea* domain, previously designated the archaebacteria (23), are divided at a fundamental level into two groups. One, the *Euryarchaeota*, encompasses methanogens and extreme halophiles, whereas the other, the *Crenarchaeota*, includes hyperthermophiles found in geothermal habitats (21). *Sulfolobus* spp. are obligately aerobic members of the latter division and grow optimally at about 80°C and pH 3 (7). Analysis of these organisms is thus motivated by interest in (i) molecular mechanisms by which biological function is maintained under otherwise denaturing conditions and (ii) fundamental aspects of ancient evolutionary lineages separate from those of popular model systems, such as *Saccharomyces cerevisiae* or enteric bacteria.

Although *Sulfolobus* spp. have been analyzed by a variety of molecular cloning and biochemical techniques, manipulation of individual molecular processes in vivo remains a serious challenge and illustrates the need for both classical and modern genetic methods. Recent progress toward genetic analysis of *Sulfolobus* spp. includes the introduction of genetic markers into wild-type strains via direct selection (7, 14). Two of the mutant phenotypes described, fluorouracil resistance (8) and fluoroorotate resistance (14) are expected to result from lesions in pyrimidine nucleotide metabolism. In the latter case, an enzymatic deficiency was identified in some of the mutants recovered (14).

Details of pyrimidine nucleotide biosynthesis have yet to be described for any extremely themophilic member of the *Archaea* domain. Other organisms synthesize UMP, the central intermediate in pyrimidine nucleotide metabolism, via six enzymatic steps, as summarized in Fig. 1. Uracil and other preformed bases can be converted to UMP by one or more additional salvage pathways (Fig. 1).

This report describes biochemical and genetic analysis of the biosynthetic pathway responsible for UMP formation in *Sulfolobus acidocaldarius*. All enzymes necessary for de novo synthesis were found in the wild-type strain, and a series of auxotrophic mutants were isolated and characterized. The results establish five gene-enzyme relationships and their metabolic significance in the cell, demonstrate the functioning of a parallel salvage pathway for the synthesis of UMP, and provide mutant strains of *S. acidocaldarius* which should be useful in future analysis of this extreme thermophile by genetic and other methods.

MATERIALS AND METHODS

***Sulfolobus* strains and growth conditions.** *S. acidocaldarius* C, strain DG6 (8), was used as the wild type. Salient properties of the mutant strains isolated in this study are summarized in Table 1; unless otherwise noted, spontaneous mutants were isolated. XT and XG growth media contained the following (in grams per liter of distilled water): K_2SO_4, 3.0; NaH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2 \cdot 2H_2O$, 0.1; sufficient H_2SO_4 to yield a pH of 3.6; and 0.02 ml of a concentrated mineral solution (5% [wt/vol] $FeCl_3 \cdot 6H_2O$ and 0.5% [each] $CuCl_2 \cdot 2H_2O$, $CoCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, and $ZnCl_2$ all in 1 M HCl). D-Xylose (0.2%) was added as the primary carbon source, while nitrogen was supplied as 0.1% Bacto Tryptone (XT medium) or 0.1% L-glutamine (XG medium). Unless otherwise noted, XT medium was used and supplemented with 10 μg of uracil per ml as indicated. Other conditions, including solidification of growth medium, were essentially as described previously (7). The density of cells in liquid suspension was calculated from the apparent A_{600} by using a standard curve; an absorbance of 1.0 corresponds to 8×10^8 cells per ml.

The ability of a compound to serve as a pyrimidine source was determined by inoculating 2.5 ml of medium containing 25 to 50 μg of the test compound with approximately 2×10^7 washed cells and by aerating the mixture at 76°C for up to 5 days. MICs were determined in microdilution plates as previously described (7). Estimates of mutation and reversion frequencies are for populations of about 10^9 cells grown

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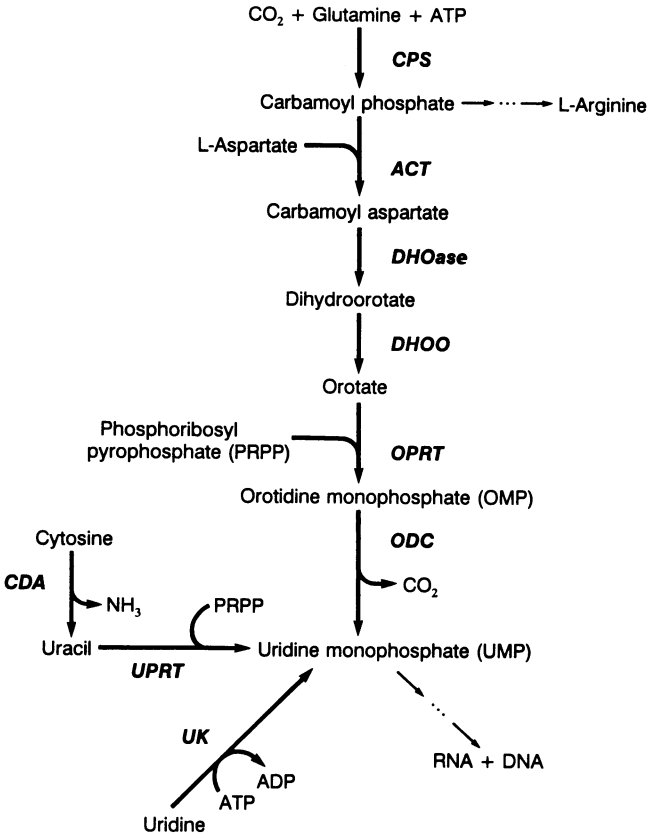

FIG. 1. General features of pyrimidine nucleotide biosynthesis. The vertical sequence depicts the de novo pathway of UMP formation, which in turn supplies RNA and DNA synthesis pathways. Alternate (i.e., salvage) routes to UMP starting from uracil, cytosine, or uridine are also shown. For a review of these and additional enzymatic conversions, see references 2, 16, and 17. CDA, cytosine deaminase; UK, uridine kinase; other abbreviations given in the text.

from isolated colonies and were measured as mutant CFU per cell plated.

Isolation of auxotrophic mutants. With the exception of *pyrE* and *pyrF* mutants, which were selected directly (see Results), auxotrophic mutants were isolated by the following procedure. A suspension of DG6 cells was irradiated with a germicidal lamp under conditions yielding less than about 1% survival; this treatment appears to increase the yield of mutant phenotypes of *S. acidocaldarius* (8). Irradiated cells were plated (no intervening growth allowed) on solid medium containing various precursors, including thymine, uracil, and hypoxanthine. The resulting colonies were screened for nutritional requirement by replica plating; putative auxotrophic colonies were streaked for isolation on supplemented solid medium and retested for nutritional requirement in liquid medium.

Genetic nomenclature. Following the recommendations of Demerec et al. (5), independent mutations yielding the same phenotype or a related phenotype were assigned serial allele numbers. Gene designations were generally assigned in conformity to those of enteric bacteria, with the exception of the *S. acidocaldarius* allele affecting carbamoyl phosphate synthetase (CPS). This locus has been designated *cbp* (carbamoyl phosphate) to distinguish it from pyrimidine-specific genes (compare with *pyrA* of *Salmonella typhimurium* [20]) and from genes responsible for synthesis of carotenoid pigments (compare with *carA* of *Escherichia coli* [1]), which *S. acidocaldarius* produces under certain conditions (7). Mutations conferring single-step resistance to several 5-fluoropyrimidines were provisionally designated *fpy* (fluoropyrimidine resistance).

Enzyme assays. When possible, assays were conducted at elevated temperatures in order to approximate physiological conditions (see below). Unless otherwise noted, the assay mixtures were buffered by 50 mM potassium 3-{[tris(hydroxymethyl)methyl]amino}propanesulfonate (pH 8.4), yielding effective pHs of approximately 7.8 and 7.5 at 55 and 70°C, respectively (3). In all the assay procedures, product accumulation was proportional to both time of incubation and volume of extract. In some cases, notably CPS, orotate phosphoribosyl transferase (OPRT), and uracil phosphoribosyl transferase (UPRT) (see below), maintaining this proportionality necessitated lower assay temperatures and limited incubation times; conditions were not otherwise optimized.

Cell extracts were prepared as follows. Cell pellets were washed in 30 mM imidazole citrate (IC buffer) (pH 6.5), resuspended in a small volume of IC buffer, and stored frozen at −20°C. Prior to use, cell suspensions were thawed and disrupted by sonication, freed of unbroken cells, and dialyzed overnight at 4°C against IC buffer. The dialyzed sonicates contained significant amounts of cell membrane, which were pelleted (1.5-ml centrifuge tubes, 10 min at 13,000 × *g*) and stored separately for use in dihydroorotate oxidase assays (see below); all extracts were stored at −70°C.

CPS (EC 2.7.2.9). Cell extract and 2.0-μmol portions (each) of $MgCl_2$, disodium ATP, [^{14}C]$NaHCO_3$ (0.1 μCi), and L-glutamine were combined at 0°C in a total volume of 0.2 ml. The reaction was initiated by immersing the tubes in a 55°C water bath and stopped by immersing the tubes in ice water; subsequent determination of [^{14}C]carbamoyl phosphate as hydroxyurea was done by the method of Levine and Kretchmer (15).

ACT (EC 2.1.3.2). Cell extract (5 to 10 μl), 3 μmol of sodium L-aspartate, and 0.6 μmol of disodium carbamoyl phosphate were combined at 0°C in a total volume of 0.30 ml. The reaction was initiated by warming the tubes to 22°C, and at regular intervals 60-μl aliquots were withdrawn and added to 0.2 ml of 0.5 M $HClO_4$. Carbamoyl aspartate was determined by the method of Prescott and Jones (19), using 2 mM sodium DL-carbamoyl aspartate (Sigma) as the standard. Enzymatic formation of carbamoyl aspartate was not observed at 55 or 70°C, probably because of its consumption by dihydroorotase (DHOase) in the crude extracts. This hypothesis is supported by the observation that the addition of crude extract suppressed the background of nonenzymatic carbamoyl aspartate formation otherwise seen in aspartate carbomyl transferase (ACT) assay mixtures incubated at 55 or 70°C.

DHOase (EC 1.3.3.1). The assay procedure was the same as for ACT above, except that aspartate and carbamoyl phosphate were replaced by 0.75 μmol of sodium L-dihydroorotate, and the assay mixture was incubated at 70°C.

Dihydroorotate oxidase (DHOO). Crude membrane preparation (5 to 10 μl) was combined with 6 μmol of sodium dihydroorotate in a total volume of 1.2 ml and incubated at 70°C. Aliquots (290 μl) were withdrawn, diluted with an equal volume of 1 M $HClO_4$, and freed of precipitate by centrifugation. Orotic acid was determined spectrophoto-

metrically (290 nm), using a net molar extinction coefficient of 6,200 (13).

OPRT (EC 2.4.2.10). Cell extract (5 to 10 μl) was added to a mixture containing 1.5 μmol of sodium orotate, 2 μmol of $MgCl_2$, and 0.055 μmol of magnesium 5-phosphophoribosyl-1-pyrophosphate (magnesium PRPP) (Calbiochem) and at 0°C in a total volume of 0.6 ml. GTP (0.06 μmol) was included in some assays (see "UPRT" below) but appeared to have no stimulatory effect. The reaction was initiated by transferring the tubes to a 55°C water bath. At 20-min intervals, the tubes were chilled, the optical density at 295 nm was measured, an additional 0.05 μmol of magnesium PRPP was added, and the tubes were reincubated. The rate of formation of orotidine monophosphate (OMP) was calculated from PRPP-dependent loss of absorbance due to the concerted action of OPRT and endogenous OMP decarboxylase in the extract (24). Extracts of *pyrF* mutants were assayed in the presence of 2-μl portions of DG96 extract (0.09 mg of protein) to ensure that OMP decarboxylase (ODC) activity was not limiting; any contribution of OPRT from this extract was later subtracted.

ODC (EC 4.1.1.23). Each assay contained 15 μmol of OMP and 3 to 6 μl of cell extract in a total volume of 0.65 ml. Rate of UMP formation was monitored as described for OPRT (above), except that the tubes were incubated at 70°C.

UPRT. Each tube contained 2 μmol of sodium *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonate as buffer (yielding pH 7.7 at 22°C), 0.4 μmol of $MgCl_2$, 0.02 μmol of magnesium PRPP, 0.04 μmol of GTP (2), and 1 μl of extract in a total volume of 40 μl at 0°C. The reaction was initiated by adding 0.012 μmol (0.33 μCi) of [5-^{3}H]uracil and incubating at 55°C. At 10-min intervals, 6-μl aliquots were applied to polyethyleneimine-cellulose thin-layer plates, and the amount of [5-^{3}H]UMP was determined after development of the chromatogram in 50% aqueous methanol (2).

Other biochemical methods. Protein concentrations were determined by a modified version (18) of Lowry's assay, using desiccated bovine serum albumin as the standard. Uptake and incorporation of exogenous uracil were measured by growing small cultures in XT medium supplemented with 5 μg of [5-^{3}H]uracil (0.16 μCi) per ml. The resulting cells were washed free of medium by repeated centrifugation and resuspension in mineral base (see above) supplemented with 0.03% gelatin. Cell density was determined as described above, and intracellular ^{3}H was measured by scintillation counting, as calibrated against a known amount of [5-^{3}H]uracil added to a suspension of nonradioactive cells.

Excreted pyrimidines were measured by the following bioassay. Stationary-phase, unsupplemented cultures of *S. acidocaldarius* were centrifuged, and aliquots of the resulting supernatants were added to tubes containing 1-ml portions of synthetic medium (Bacto Yeast nitrogen base [Difco] supplemented with glucose [0.5%], L-histidine [100 μg/ml], L-methionine [100 μg/ml], and L-tryptophan [30 μg/ml]) containing about 10^5 cells of a *ura3* strain (JM43) of *S. cerevisiae*. After 2 days of incubation at 30°C, growth was measured turbidometrically and compared with a standard curve. Under these conditions, uracil and uridine gave similar growth responses on a molar basis; the detection limit corresponded to about 15 ng of uracil.

RESULTS

Enzymatic activities of UMP biosynthesis in wild-type *S. acidocaldarius*. Dialyzed, cell extracts of *S. acidocaldarius* DG6 were assayed for enzymes of the de novo and salvage pathways of UMP biosynthesis. Under the assay conditions used (see Materials and Methods), the following enzymes and activities (in nanomoles per minute per milligram of protein) were found: CPS, 0.43; ACT, 0.15; DHOase, 0.66; DHOO, 0.063; OPRT, 0.98; and ODC, 6.4. CPS was assayed as the glutamine-utilizing activity; it was not established whether ammonium could also serve as a substrate. Formation of carbamoyl aspartate by ACT was apparent at 22°C but not at 55 or 70°C in crude extracts (see Materials and Methods). DHOO activity was found in crude cell membrane preparations, but not in cytosolic fractions.

These results demonstrated that *S. acidocaldarius* has the enzymatic activities necessary to synthesize UMP de novo via orotic acid like other organisms (Fig. 1). Many organisms can also synthesize UMP from preformed bases or nucleosides. Direct enzymatic evidence for such a pyrimidine salvage pathway in *S. acidocaldarius* was provided by detection of UPRT activity in dialyzed extracts (specific activity of 1.2 nmol/min/mg of protein). Additional evidence in vivo includes the observed toxicity of 5-fluorouracil (FUR) for strain DG6 (8) and the ability of free uracil to support growth of its pyrimidineless derivatives (see below).

Isolation of pyrimidine-requiring mutant strains of *S. acidocaldarius*. Selection of spontaneous mutants resistant to 5-fluoroorotic acid (FOR) in the presence of uracil was found to be a convenient and effective means of isolating pyrimidine auxotrophs of *S. acidocaldarius* DG6 (also see reference 14). In the present study, spontaneous For^r mutants were typically recovered at frequencies of 1×10^{-7} to 3×10^{-7} per cell plated on XT medium containing 50 μg of FOR per ml and 10 μg of uracil per ml. In other organisms, the basis of For^r selection is toxicity of the 5-fluoro-UMP formed from fluoroorotate, so that the auxotrophs selected are those lacking biosynthetic conversions between orotate and UMP (Fig. 1). By analogy, two *S. acidocaldarius* enzymes could be expected to be inactivated among the auxotrophic For^r mutants, i.e., OPRT and ODC. Phenotypic characterization (see below) suggested that two distinct classes of auxotrophic (e.g., Pyr$^-$) mutants were indeed isolated by For^r selection. One representative strain of each phenotypic class, designated DG66 and DG96, was therefore chosen for further study. In order to obtain mutants lacking earlier steps of the pathway, a brute-force screening procedure was applied to approximately 20,000 colonies grown from UV-treated *S. acidocaldarius* cells (see Materials and Methods). Three mutant strains, DG29, DG38, and DG64, were recovered that exhibited a Pyr$^-$ phenotype, i.e., that required a pyrimidine compound for growth (Table 1).

Five *S. acidocaldarius* strains (DG66, DG96, DG29, DG38, and DG64) shared the property that sustained growth in XT or XG medium required either uracil or cytosine; thymine, adenine, or hypoxanthine failed to support growth. Uracil supported growth better than cytosine did, whereas the pyrimidine nucleosides uridine and cytidine were poor pyrimidine sources for the auxotrophs, although some growth was occasionally observed. Under most conditions, uracil at initial concentrations of about 2 μg/ml limited the final growth yields.

Enzymatic activities of Pyr$^-$ strains. To identify the biochemical lesions of the above mutant strains, cell extracts of the mutants and of the available Pyr$^+$ revertants were assayed for enzymes of de novo UMP biosynthesis. Each of the auxotrophic strains DG38, DG64, DG29, and DG96 were found to lack only one of the six enzymatic activities, and in the corresponding phenotypic revertants, the activity was

TABLE 1. *S. acidocaldarius* strains used in this study

Strain	Phenotype	Genotype	Derivation or reference
DG6		Wild-type	8
DG15	Fpy^r	*fpy-4*	8
DG29	Pyr$^-$	*pyrD1*	UV treatment of DG6
DG38	Pyr$^-$Arg$^-$	*cbp-2*	UV treatment of DG6
DG64	Pyr$^-$	*pyrB4*	UV treatment of DG6
DG66	Pyr$^-$For^r	*pyrF5*	For^r selection of DG6
DG69	Pyr$^-$For^r	*pyrB4 pyrF7*	For^r selection of DG64
DG70	Fpy^r	*fpy-5*	Fud^r selection of DG6
DG77	Fpy^r	*fpy-10*	Pyr$^+$ For^r selection of DG6
DG82	Fpy^r	*fpy-11*	Fcy^r selection of DG6
DG86	Pyr$^+$		Revertant of DG64
DG87	Pyr$^+$		Revertant of DG66
DG93	Pyr$^+$Arg$^+$		Revertant of DG38
DG96	Pyr$^-$For^r	*pyrE9*	For^r selection of DG6
DG99	Pyr$^+$		Revertant of DG96
DG101	Fpy^r	*fpy-18*	Fcy^r selection of DG6
DG103	Fpy^r	*fpy-19*	Fud^r selection of DG6
DG104	Fpy^r	*fpy-20*	Fud^r selection of DG6
DG105	Fpy^r	*fpy-21*	Fur^r selection of DG6
DG106	Fpy^r	*fpy-22*	Fur^r selection of DG6
DG107	Fpy^r	*fpy-23*	Fur^r selection of DG6

restored (Table 2). Strain DG66 (and two phenotypically identical, independent mutants, see below) was found to lack two activities: ODC and OPRT, both of which were restored in the Pyr$^+$ revertant (Table 2). In all, five of the six enzymes leading to the formation of UMP (the de novo pathway) were found to be individually affected by mutation among the *S. acidocaldarius* auxotrophs isolated in this study (Table 2).

The results thus established the following allele-enzyme relationships: *cbp-2* impairs CPS, *pyr-3* inactivates ACT, *pyr-1* inactivates DHOO, *pyr-9* inactivates OPRT, and *pyr-5* inactivates ODC and OPRT. In addition, it was evident that under the growth conditions used, auxotrophic strains exhibited higher levels of certain enzymes (notably CPS, ACT, DHOase, and DHOO) than wild-type or revertant strains (Table 2). This result suggested induction or derepression of enzyme synthesis in vivo, presumably triggered by the onset of kinetic limitation of growth as the pyrimidine supplement was consumed.

Metabolic consequences of the enzymatic deficiencies. Although strain DG38 exhibited an unambiguous requirement for pyrimidine compounds, it exhibited a significant residual level of CPS (Table 2). Eukaryotes typically contain two separate CPS species, whose product does not intermingle in vivo, one for UMP biosynthesis and one for arginine biosynthesis (12). Inactivation of only one of two CPS species could not account for the residual CPS activity in *S. acidocaldarius*, however, as shown by phenotypic and reversion behavior of strain DG38. In addition to its pyrimidine requirement, strain DG38 showed an unconditional requirement for arginine. This dual auxotrophy implies a complete inability to synthesize carbamoyl phosphate in vivo (17; also see Fig. 1). Furthermore, reversion properties of strain DG38 indicated that the two nutritional requirements result from inactivation of only one gene. The frequency of simultaneous phenotypic reversion, i.e., recovery of Arg$^+$ Pyr$^+$ colonies, was about the same as the frequency of individual reversion to either Arg$^+$ or Pyr$^+$ alone, i.e., 0.5×10^8 to 5×10^8 per cell plated. In addition, scoring of several independent single revertants showed that in each case the unselected phenotype had simultaneously reverted. Finally, restoration of CPS activity in a phenotypic revertant of strain DG38 (Table 2) shows that the CPS deficiency of DG38 is responsible for its dual auxotrophy. The affected gene product is thus necessary for the function of the CPS of *S. acidocaldarius*. (Other possible causes of the apparent CPS activity in DG38 extract remain to be investigated. Although inactive in vivo, for example, the mutant enzyme may have been partially active under assay conditions because of the lower temperature and higher concentrations of substrates.)

The metabolic consequences of all five *pyr* mutations were further investigated on a phenotypic basis by determining each strain's ability to utilize various supplements, as well as its level of acquired resistance to FOR (Table 3). Strains DG38 and DG64 shared the ability to utilize exogenous carbamoyl aspartate and subsequent intermediates of the de novo pathway as the sole pyrimidine source. This result corroborated enzymatic evidence of defects early in the pathway, i.e., at or before the formation of carbamoyl aspartate. This result also further demonstrated that all nonphosphorylated intermediates of the de novo pathway are reasonably stable under the low pH and high temperature used for *Sulfolobus* culture and that *S. acidocaldarius* can normally obtain all such compounds from the growth me-

TABLE 2. Enzymatic activities of *S. acidocaldarius* auxotrophs and revertants

Strain (phenotype)[a]	Specific activity (% of wild-type activity)[b]					
	CPS	ACT	DHOase	DHOO	OPRT	ODC
DG6 (wild type)	0.43 (100)	0.15 (100)	0.66 (100)	0.063 (100)	0.98 (100)	6.4 (100)
DG38 (Pyr$^-$ Arg$^-$)	**0.19 (44)**	0.36 (240)	0.86 (130)	0.140 (220)	1.07 (110)	6.8 (105)
DG93 (Pyr$^+$ Arg$^+$)	1.37 (320)	0.35 (230)	0.64 (97)	0.080 (130)	1.00 (100)	7.5 (120)
DG64 (Pyr$^-$)	0.50 (115)	**0.01 (7)**	2.39 (360)	0.295 (470)	0.87 (89)	5.6 (87)
DG86 (Pyr$^+$)	0.66 (150)	0.16 (106)	0.64 (97)	0.061 (97)	1.00 (100)	8.3 (130)
DG29 (Pyr$^-$)	1.40 (330)	1.25 (820)	2.08 (315)	**<0.002 (<3)**	0.87 (89)	6.5 (101)
DG96 (Pyr$^-$)	0.98 (230)	0.85 (560)	1.91 (290)	0.189 (300)	**0.12 (13)**	9.4 (145)
DG99 (Pyr$^+$)	0.75 (170)	0.14 (89)	0.76 (115)	0.046 (73)	0.94 (96)	7.7 (120)
DG66 (Pyr$^-$)	1.68 (390)	1.52 (1,000)	2.28 (345)	0.215 (340)	**<0.02 (<2)**[c]	**0.6 (10)**
DG87 (Pyr$^+$)	0.50 (115)	0.16 (103)	0.56 (85)	0.066 (105)	0.98 (100)	6.8 (106)

[a] Cultures were grown in XT medium containing 5 μg of uracil per ml (see Materials and Methods). Each strain designated Pyr$^+$ is a phenotypic revertant of the preceding auxotroph; the derivation and genetic nomenclature of strains are summarized in Table 1.

[b] Specific activity is measured in nanomoles per minute per milligram of protein (see Materials and Methods) is an average of four determinations agreeing within approximately 25%. Activities affected by mutation are shown in boldface type.

[c] Assayed in the presence of DG96 extract (see Materials and Methods).

TABLE 3. Growth of auxotrophic strains on various pyrimidine supplements

Strain	FOR MIC (μg/ml)[a]	Growth on the indicated supplement[b]:					
		None	CbAsp	H_2-Oro	Oro	Ura	Cyt
DG6	8	+	ND	+	+	+	ND
DG38	8	0	+	+	+	+	+
DG64	8	0	+	+	+	+	+
DG29	8	0	0	0	+	+	+
DG96[c]	180	+	+	+	+	+	+
DG66	>1,000	0	0	0	0	+	+
DG69	>1,000	0	0	0	0	+	ND

[a] MICs were determined in XT medium containing uracil (see Materials and Methods).

[b] XT and XG media (two trials) supplemented with 20 μg of the indicated compound per ml were inoculated with about 2×10^7 washed cells, and turbidity was monitored for 2 to 5 days. Symbols: 0, no growth; +, growth to high density (i.e., about 3×10^8 cells per ml). Abbreviations: ND, not determined; CbAsp, carbamoyl aspartate; H_2-Oro, dihydroorotate; Oro, orotate; Ura, uracil; Cyt, cytosine.

[c] See text for discussion of residual growth of *pyrE* mutants.

TABLE 4. Resistance levels of *S. acidocaldarius* fluoropyrimidine-resistant (*fpy*) mutants to various pyrimidine analogs

Selective agent(s)[a]	Strain	Observed MIC (μg/ml)[b]			
		FUR	FUD	FCY	FOR
None	DG6	15	260	120	8
FUR	DG105	90	940	380	25
FUR	DG106	≥2,000	10,000	>2,000	190
FUR	DG107	125	5,000	1,000	45
FUR[c]	DG15	2,000	>4,000	1,500	30
FUD	DG70	60	1,000	400	15
FUD	DG103	≥2,000	10,000	2,000	125
FUD	DG104	125	940	750	45
FCY	DG82	30	1,000	400	15
FCY	DG101	125	940	250	25
FOR	DG77	2,000	4,000	2,000	60
FOR + Ura	DG66	60	500	250	>1,000

[a] Except where noted, selections employed 30 μg of FUR per ml, 300 μg of FUD per ml, 200 μg of FCY per ml, or 50 μg of FOR per ml.

[b] MICs determined as described in footnote *a* of Table 3.

[c] Strain DG15 was isolated with 150 μg of FUR per ml.

dium. This latter property is not shared by *S. cerevisiae* or enteric bacteria (16) and greatly simplifies phenotypic analysis of *Sulfolobus* pyrimidine auxotrophs.

Strain DG29 was found to utilize orotate but not dihydroorotate (Table 3), providing independent evidence for the loss of DHOO in this strain. In agreement with their ability to utilize orotate, strains DG38, DG64 and DG29 were sensitive to FOR. In contrast to the above strains, mutants selected as For^r (e.g., strains DG96 and DG66) could not grow on orotate or its preceding intermediates (Table 3). This result was in agreement with the enzymatic data showing the loss of OPRT or of ODC and OPRT in these strains (Table 2). Washed DG96 cells grew for several generations in liquid medium after the removal of supplements (Table 3) but did not subsequently form colonies in the absence of pyrimidine supplementation (Fig. 2). The auxotrophic requirement of strain DG96 was also confirmed by the isolation of a phenotypic revertant and by the failure of strain DG96 (but not of its revertant) to yield Fpy^r mutants (see below for explanation). The auxotrophy of strain DG96 could thus be characterized as somewhat leaky, and in agreement with this observation, this strain exhibited an intermediate level of FOR resistance (MIC of 200 μg/ml). These properties were in contrast to those of strain DG66, which exhibited a stringent Pyr$^-$ phenotype and high levels of FOR resistance (MIC > 1,000 μg/ml).

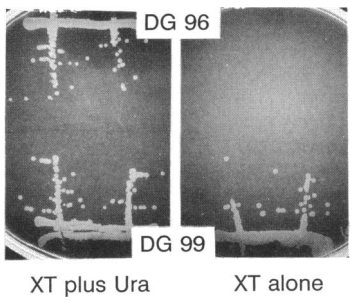

FIG. 2. Pyrimidine requirement of strain DG96. Unsupplemented cultures of Table 3 were streaked on solid XT medium (9-cm-diameter plates) containing or lacking 10 μg of uracil (Ura) per ml as shown and incubated for 1 week.

Isolation of several independent mutants showed that the enzymatic and phenotypic properties of strains DG96 and DG66 are not allele specific but typify *S. acidocaldarius pyrE* and *pyrF* mutants, respectively. Several independent mutants of each class were characterized and found to have the same phenotypes with respect to residual growth after the removal of pyrimidine and the same MIC for FOR. Enzymatic assay of two independent *pyrE* mutants and two independent *pyrF* mutants revealed the same deficiencies as in DG96 and DG66, respectively. It should be noted that in addition to mutants of the *pyrE* and *pyrF* classes, a third class of For^r mutants that did not require pyrimidine compounds for growth was recovered (see below).

Mutations conferring general resistance to 5-fluoropyrimidines. The above result that fluoroorotate selects mutants lacking either OPRT (*pyrE*) or ODC and OPRT (*pyrF*) indicated that in *S. acidocaldarius*, as in other organisms, the 5-fluoro analog is metabolized in the same way as orotate and becomes deleterious only after conversion to 5-fluoro UMP (16). Similarly, blocking the formation of UMP from preformed nucleobases or nucleosides by inactivation of salvage enzymes would be expected to confer resistance to the corresponding 5-fluoro analogs (2). Indeed, the sensitivity of strain DG6 to FUR and the selection of spontaneous Fur^r mutants have been described previously (8). One plausible explanation for these mutants is the loss of UPRT activity (2).

To attempt selection of additional defects in pyrimidine salvage functions, 5-fluorocytosine (FCY) and 5-fluorouridine (FUD) were tested and found to inhibit growth of *S. acidocaldarius*, albeit with higher thresholds (MICs) than for FOR or FUR (Table 4). Spontaneous resistance mutants were readily obtained by plating DG6 cells onto solid medium containing any of the above compounds at a concentration above the observed MIC. The For^r mutants so obtained, represented by strain DG77 (Table 4), were prototrophs, as expected from the absence of a pyrimidine source in the selective medium. Such mutants were also obtained on uracil-supplemented medium containing FOR but were usually outnumbered by auxotrophic (*pyrE* and *pyrF*) mutants under those selection conditions.

Phenotypic characterization of several independent mutants selected as Fur^r, Fud^r, Fcy^r, or For^r showed that none

TABLE 5. Other properties of *S. acidocaldarius* fluoropyrimidine-resistant (*fpy*) mutants[a]

Strain	FUR MIC (μg/ml)[b]	% Incorporation (wt) of exogenous uracil	UPRT activity (nmol/min/mg)[c]	% Excretion (wt) of pyrimidines
DG6	15	100[d]	1.18 ± 0.03	100[e]
DG105	90	41 ± 3	1.68 ± 0.13	620 ± 200
DG107	125	26 ± 5	1.99 ± 0.05	450 ± 50
DG15	2,000	15 ± 4	1.74 ± 0.20	1,650 ± 100
DG106	≥2,000	7.8 ± 0.4	1.57 ± 0.44	890 ± 360
DG70	60	40 ± 6	ND[f]	420 ± 130
DG104	125	44 ± 11	1.63 ± 0.37	1,300 ± 320
DG103	≥2,000	10 ± 2	ND	2,800 ± 800
DG82	30	31 ± 8	ND	270 ± 130
DG101	125	48 ± 13	1.50 ± 0.16	330 ± 130
DG77	2,000	13 ± 3	ND	1,100 ± 150

[a] Unless noted, data are means ± half the range of two determinations.
[b] Data of Table 4, included for reference.
[c] For details, see Materials and Methods.
[d] This value corresponds to 896 ng of uracil per 10^9 cells.
[e] This value corresponds to 444 ng of uracil per 10^9 cells.
[f] ND, not determined.

of the acquired resistances was specific to the compound used for selection. Whereas the auxotrophic For^r (i.e., *pyrE* and *pyrF*) mutants described above had acquired resistance to only FOR, the prototrophic mutants showed increased resistance to all fluoropyrimidines tested (Table 4). In addition, the relative levels of this generalized resistance varied considerably among the mutants characterized, in contrast to the strictly bimodal distribution of FOR resistance levels among *pyrE* and *pyrF* mutants (Table 3). Phenotypic criteria thus did not support the division of prototrophic, Fpy^r mutants into distinct subclasses.

Genetic and biochemical properties of Fpy^r mutants. The most notable genetic property of Fpy^r mutants was apparent incompatibility of the Fpy^r and Pyr$^-$ phenotypes. Whereas Fpy^r mutants of Pyr$^+$ strains were recovered at frequencies of up to 10^{-5} per cell plated, no Fpy^r derivative of DG64 could be isolated, despite repeated attempts with medium supplemented with uracil, cytosine, orotic acid, or carbamoyl aspartate. Furthermore, colonies which could be isolated from strain DG29 or DG38 plated on 30 μg of FUR per ml did not exhibit significant fluoropyrimidine resistance, as shown by MIC determination in liquid medium (9).

Efforts to identify the biochemical lesion(s) responsible for the Fpy^r phenotype are summarized in Table 5. All mutant strains were deficient in uptake and incorporation of exogenous uracil, and the magnitude of the defect generally correlated with the level of fluorouracil resistance. This effect could not be attributed to the loss of UPRT, which was present at wild-type levels in all of the various Fpy^r mutants assayed. However, all representative Fpy^r mutants were found to excrete pyrimidines into the growth medium (Table 5). The amount excreted generally correlated with the observed impairment of uracil uptake and incorporation and also correlated with the observed increase in fluorouracil resistance over that of the wild type. These results suggest that the overproduction of endogenous pyrimidine compounds may compete with incorporation of exogenous pyrimidine analogs, causing the observed Fpy^r phenotype. This phenomenon has been observed for mutant strains of other organisms and has in some cases been traced to induction or derepression of de novo biosynthetic enzymes (16). The levels of two apparently regulated enzymes, ACT and DHOase, in strains DG15, DG101, DG104, DG105, and DG106 were found to be normal, however (0.18 to 0.25 and 0.69 to 0.95 nmol/min/mg of protein, respectively).

DISCUSSION

The present work establishes that *S. acidocaldarius* synthesizes pyrimidine nucleotides via the intermediates carbamoyl phosphate plus aspartate, carbamoyl aspartate, dihydroorotate, orotate, OMP, and UMP. This conclusion is supported by the following: (i) detection of significant levels of all necessary enzymatic activities in extracts of prototrophic strains, (ii) isolation of pyrimidine-requiring mutants whose growth is supported by one or more of the above intermediates, (iii) loss of only one of the necessary enzymatic activities (with the exception of *pyrF* mutants) in a given auxotroph, and (iv) restoration of the lost activity (or activities) upon reversion to the Pyr$^+$ phenotype. Among the *S. acidocaldarius* strains described here, five of the six enzymes of the de novo pyrimidine nucleotide pathway have been impaired by mutation, and the metabolic consequences have been characterized, making de novo UMP biosynthesis in strain DG6 one of the genetically best-documented biochemical pathways of the *Archaea* to date.

The genetic evidence demonstrates that *S. acidocaldarius* has a single CPS common to both the UMP and arginine pathways; this situation is found in enteric bacteria, whereas *Bacillus subtilis* and fungi have two CPS species (16). In enteric bacteria, the dependence of UMP and arginine biosynthesis on a shared intermediate has been exploited in the genetic analysis of regulatory circuits (16) and may have similar utility in *Sulfolobus* spp. Indeed, the present study provides the first direct evidence of pathway regulation in a crenarchaeote. Levels of the early enzymes of UMP biosynthesis assayed in extracts were 3- to 10-fold higher for auxotrophic strains than for the wild type or Pyr$^+$ revertants. Because the cultures were supplemented with 5 μg of uracil per ml, these ratios probably represent conservative estimates of inducibility of the corresponding enzymes. This hypothesis is supported by the results of ACT assays in uracil-starved DG66 cells, which show higher induction ratios than those of Table 2 (9).

The facile recovery of pyrimidine auxotrophs following selection with fluoroorotate plus uracil is a useful property that *Sulfolobus* spp. share with other organisms. This property has been independently observed by Kondo et al. (14), who isolated For^r mutants of *Sulfolobus* sp. strain 7 and found strains deficient in OPRT among the resulting isolates. The present study showed that selection with less than about 100 μg of FOR per ml supplemented with uracil results in three distinct classes of *S. acidocaldarius* mutants: generalized Fpy^r mutants (see below), *pyrE* mutants, and *pyrF* mutants. Each class can be distinguished by its growth phenotype. Fpy^r mutants display a range of apparent resistance to FOR but are readily identified by their prototrophy and elevated resistance to other fluoropyrimidines. Mutants of the *pyrF* class lack both OPRT and ODC, and exhibit high resistance to FOR (MIC greater than 1,000 μg/ml) and a stringent requirement for pyrimidines. In contrast, mutants of the *pyrE* class, which lack only OPRT activity, exhibit moderate resistance to FOR (MIC less than about 200 μg/ml) and a leaky auxotrophic phenotype. The simultaneous loss of both ODC and OPRT in *pyrF* mutants and the characteristic leakiness (bradytrophy) of *pyrE* mutants seem significant but remain to be elucidated in molecular terms. The

ODC and OPRT activities may reside on a single polypeptide, for example, in which mutation of the ODC domain often disrupts or removes the OPRT domain, but not vice versa.

Results of the present study also establish the functioning of at least one salvage route for the synthesis of pyrimidine nucleotides in *S. acidocaldarius*. The existence of a salvage route was demonstrated by the toxicity of FUR and FCY, the efficient utilization of uracil or cytosine as a pyrimidine source by all Pyr^- auxotrophs, and the detection of UPRT activity in cell extracts from this extreme thermophile. Efforts to isolate spontaneous mutants lacking one or more salvage functions yielded mutants with elevated resistance to several 5-fluoropyrimidines, rather than mutants specifically resistant to one analog. As predicted by their generalized resistance, these *fpy* mutants could be selected by any of several fluoropyrimidines. The biochemical basis for the phenotype is not the loss of UPRT activity (compare with *upp* mutants of *E. coli* [2]), but it does appear to involve overproduction and excretion of one or more pyrimidine compounds. This property is reminiscent of certain *pyrH* (UMP kinase-deficient) mutants of *Escherichia coli*, which exhibit abnormally high expression of ACT and other regulated de novo pathway enzymes (16).

The exact mechanism of pyrimidine overproduction in *Sulfolobus fpy* mutants remains to be determined. ACT and DHO levels were normal in these strains, although it has not been established that either enzyme is rate limiting in vivo. Alternatively, *fpy* mutations may affect regulation of catalysis rather than regulation of enzyme levels; these mutations could, for example, abolish allosteric inhibition of ACT or some other key enzyme of the pathway. Characterization and purification of the pyrimidine nucleotide biosynthetic enzymes may shed light on this question. Other properties of the *Sulfolobus* mutants must also be considered in further investigations of their biochemical basis. The high spontaneous frequency of the phenotype (about 10^{-5} per cell plated) suggests a simple loss of function, rather than a change in enzymatic specificity, yet a range of resistance levels is obtained among the spontaneous mutants. It also seems significant that the Fpy^r phenotype appears incompatible with loss of de novo UMP biosynthesis, despite simultaneous supplementation with utilizable intermediates of the de novo pathway, such as orotate.

The de novo route to UMP is a well-defined enzymatic sequence of central metabolic importance. This route is essential for genome replication and gene expression and supplies a significant fraction of the prokaryotic cell mass. In addition, this route has historically served as an instructive model system for studying mechanisms of catalytic and genetic control, both in *Bacteria* and in *Eucarya* (eukaryotes) (23). The fact that *S. acidocaldarius* uses the same enzymatic activities as unrelated groups of organisms argues that this is indeed a universal and ancient pathway (17). Members of the domains *Bacteria* and *Eucarya* nevertheless exhibit fundamental differences with respect to the regulation and the molecular forms of certain UMP biosynthetic activities. Members of the *Bacteria* domain have separate polypeptides encoded by separate (and often unlinked) genes, whereas members of the *Eucarya* domain encode CPS and ACT (and in some cases additional enzymatic activities) as a single polyfunctional gene product (22). It will therefore be of interest to determine whether the apparently independent mutability and regulation of CPS and ACT activities of *S. acidocaldarius* actually reflect two separate genes, and conversely, whether OPRT and ODC of *S. acidocaldarius* are derived from a single polypeptide, as is apparently the case in animal cells (12).

Finally, it should be noted that the results of this study provide the basis for developing additional genetic techniques for *Sulfolobus* species in the future. The auxotrophic mutants described here exhibited relatively low rates of phenotypic reversion and in principle provide strong, positive selection for the introduction of DNA encoding the corresponding functional enzyme. The potential of these selectable markers for the development of transformation methods and cloning vectors is considerable, as illustrated by the genetic manipulations of *S. cerevisiae* made possible by use of *URA3* and other biosynthetic genes (4, 11). In addition, the ability to select the *pyrF* class of mutants exclusively by an appropriate choice of conditions should facilitate quantitative analysis of spontaneous mutation in *Sulfolobus* species. Such an approach has yielded preliminary estimates of the rate of forward mutation at the *S. acidocaldarius pyrF* locus (9). Although the effects of phenotypic lag and other factors have yet to be defined, the uncorrected rate, on the order of 2×10^{-7} mutational events per cell per generation at 74°C (10) is less than or equal to corrected rates of forward mutation reported for typical *E. coli* and *S. cerevisiae* loci (6). Thus, *S. acidocaldarius* seems to adequately compensate for the increased rates of spontaneous DNA damage expected at its elevated growth temperature. Since this organism grows well at temperatures above 80°C (7), it would be of interest to investigate the temperature dependence of DNA replication fidelity in this extreme thermophile, as well as some of the genes necessary to maintain this fidelity.

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