Global Responses of *Methanococcus maripaludis* to Specific Nutrient Limitations and Growth Rate^{∇†}

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Continuous culture, transcriptome arrays, and measurements of cellular amino acid pools and tRNA charging levels were used to determine the response of *Methanococcus maripaludis* to leucine limitation. For comparison, the responses to phosphate and H₂ limitations were measured as well. In addition, the effect of growth rate was determined. Leucine limitation resulted in a broad response. tRNA^{Leu} charging decreased, but only small increases in mRNA were seen for amino acid biosynthesis genes. However, the cellular levels of free isoleucine and valine showed significant increases, indicating a coordinate regulation of branched-chain amino acids at a post-mRNA level. Leucine limitation also resulted in increased mRNA abundance for ribosomal protein genes, increased rRNA abundance, and decreased mRNA abundance for genes of methanogenesis. In contrast, phosphate limitation induced a specific response, a marked increase in mRNA levels for a phosphate transporter. Some mRNA levels responded to more than one factor; for example, transcripts for flagellum synthesis genes decreased under conditions of leucine limitation and increased under H₂ limitation. Increased growth rate resulted in increased mRNA levels for ribosomal protein genes, increased rRNA abundance, and increased mRNA for a gene encoding an S-layer protein.

In a variety of organisms, nutrient limitation elicits both specific and global regulatory responses. In the *Bacteria*, amino acid limitation can increase gene expression and enzyme activities associated with a specific pathway. On the other hand, a wide variety of bacteria employ the stringent response, a global response to amino acid limitation, whose effects include a dramatic decrease in the accumulation of stable RNA (2). Amino acid limitation has been less well studied in the *Archaea*, but both pathway-specific and global responses have been found. An example of a pathway-specific response is found in the regulation of the *trp* operon by tryptophan in *Methanothermobacter thermautotrophicus*, mediated by the TrpY repressor (12, 34). On the global level, a subset of archaeal species possesses a stringent response; however, they do not employ the guanosine tetra- and pentaphosphates characteristic of bacterial systems, and the mechanism of stringency in archaea remains unknown (3, 5, 26).

In addition to responding to nutrient limitation, many organisms calibrate their molecular and metabolic functions to variations in growth rate. In *Escherichia coli*, the correlation between growth rate and the number of ribosomes per cell is well known, and ribosomal content is generally proportional to the growth rate. Ribosomal protein mRNA levels vary as well (17, 22). Similarly, in *Saccharomyces cerevisiae*, ribosomal protein responds to growth rate at the translational and mRNA

abundance levels (18, 22). Similar studies with the *Archaea* have been limited. In the archaeon *Pyrococcus furiosus*, 16S rRNA abundance, measured as a fraction of total RNA, increased with growth rate (7), although not in equal proportions.

Methanococcus maripaludis is a species of archaea representative of the hydrogenotrophic methanogens, which gain energy by using H₂ to reduce carbon dioxide to methane. *M. maripaludis* has served as a model species for studies of specific regulatory mechanisms (9, 19) as well as global studies at the transcriptome and proteome levels (14, 23, 33). Our approach to the global study of nutrient limitation and growth rate relies on continuous cultures grown in chemostats. For nutrient limitation studies, growth rate and cell density are held constant, while the nutrient limiting the growth is varied. For a given nutrient limitation, at least two comparisons are made, each to a different nutrient limitation. Thus, the study of leucine limitation necessitates the presentation of comparative data for phosphate limitation and H₂ limitation. For growth rate studies, the cell density and the limiting nutrient are held constant. A previous report details the effects of H₂ limitation and growth rate on mRNA levels for genes of methanogenesis and carbon dioxide assimilation, using a similar approach (14). Here, we focused on the response to more broadly applicable nutrient conditions, limitations for the amino acid leucine and phosphate, and the response to varying growth rate. We measured the effects on mRNA levels, cellular amino acid pools, and tRNA charging.

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MATERIALS AND METHODS

Continuous culturing, RNA extraction, and array analysis. *M. maripaludis* strain S52 (13), a leucine auxotroph derived from the wild-type strain S2 (31),

contains a disruption of *leuA* (Mmp1063), which encodes isopropylmalate synthase. It was grown by continuous culture in McA, a defined, rich medium containing acetate, amino acids, and aryl acids, which were provided in two pools, designated amino acid stock 1 and amino acid stock 2, and minerals and other nutrients (13). Growth conditions, cell harvesting, RNA extraction, real-time reverse transcription (RT)-PCR, and array analyses were as described previously (14). For nutrient limitation studies, growth rate and cell density were held constant at a specific growth rate of 0.125 h^{-1} and an optical density at 660 nm (OD_{660}) of approximately 0.6. For studies of growth rate effects, two growth rate comparisons were conducted, one in which H_2 was the limiting nutrient, and one in which phosphate was the limiting nutrient. A specific growth rate of 0.042 h^{-1} was compared with either 0.19 h^{-1} (in the experiment where phosphate was limiting) or 0.2 h^{-1} (in the experiment where H_2 was limiting). Cell density was held constant at an OD_{660} of approximately 0.6. Arrays were conducted with four (nutrient limitation comparisons) or three (growth rate comparisons) biological replicates for each condition. Each comparison involved four technical replicates as described previously (33). Gene expression ratios were viewed using a TIGR MultiExperiment viewer (24). Data are available at the NCBI Gene Expression Omnibus (GEO) through accession numbers GSE6747 and GSE8728. 16S rRNA abundance was calculated by multiplying the yield of total RNA (μg per OD_{660} per ml of culture) by 0.24. Real-time RT-PCR results indicated that the percentage of the total RNA attributable to 16S rRNA was invariant, and the value of 24% was obtained from RNA analyses done with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Amino acid analysis. For each nutrient-limited condition, a chemostat culture was allowed to equilibrate to steady state, cells were harvested, and the procedure was repeated to obtain two replicate samples. Each sample was obtained as follows. Twenty milliliters of culture at an OD_{660} of ~ 0.6 was transferred into a prechilled 1-liter round-bottomed flask and rapidly cooled to $\sim 10^\circ\text{C}$. Three 5-ml portions were transferred into 15-ml conical screw-cap tubes, and the cells were then collected by centrifugation at $11,000 \times g$ at 4°C . The cell pellets were then washed by mixing them with 10 ml of prechilled nitrogen-free medium (1) and immediately re-collected by centrifugation at $17,000 \times g$ for 5 min at 4°C . The cell pellets were then frozen in a dry ice-ethanol bath and stored at -82°C . For amino acid analysis, the two replicate samples collected from 30 ml (total) of culture were combined. The cells were thawed with 1 ml of 0.1 M HCl with 9.7 μM of β -aminoisobutyric acid as an internal standard. After samples were lysed, they were centrifuged at $16,100 \times g$ for 10 min (at room temperature) to remove debris. The cell extracts were then lyophilized before being sent for amino acid analysis.

For control measurements, *M. maripaludis* strain S2 was grown in batch culture with 100 ml McNA medium (McN medium [31] with 1.36 g/liter sodium acetate $\cdot 3\text{H}_2\text{O}$), using 0.5 g mercaptoethanesulfonic acid as a reducing agent. The culture was grown to an OD_{660} of 0.45 and chilled on ice. Three 30-ml portions of culture were processed separately. To estimate the carryover of amino acids from the medium, α -amino-*n*-butyric acid was added to each portion to a final concentration of 1 mM. The cells were then collected by centrifugation at $12,100 \times g$ for 10 min at 4°C , washed once with 30 ml of McN medium, and re-collected by centrifugation at $12,100 \times g$ for 5 min at 4°C . Cells were lysed and prepared for amino acid analysis as described above. For the control measurements, the carryover of amino acids from the medium was not detectable.

The samples were sent to the Molecular Structure Facility at the University of California, Davis, for amino acid analysis using a Beckman 6300 Li citrate-based amino acid analyzer. Each sample was dissolved in 100 μl of 0.1 M HCl with 2% sulfosalicylic acid. Then, 100 μl of dilution buffer containing 10 nmol of aminomethyl cysteine (AE-Cys) was added. AE-Cys was run as an internal standard for the operation of the analyzer over time. For the quantification of glutamate and aspartate, samples were diluted 20-fold.

Extraction and analysis of tRNAs. Cell pellets from 250 ml of chemostat culture were processed based on the method described in reference 4. Each sample was resuspended in 10 ml of lysis buffer (0.3 M sodium acetate [pH 5.0], 10 mM EDTA), and 10 ml of phenol saturated with lysis buffer was added. The suspensions were vortexed for 5 min and centrifuged at 7,000 rpm for 15 min. All centrifugation steps were carried out in an SS-34 rotor in a Sorvall RC-5 centrifuge. The aqueous phase was transferred to a new tube, and isopropanol was added to 20% (vol/vol). The mixture was centrifuged at 7,000 rpm for 15 min, supernatant was transferred to a new tube, and additional isopropanol was added to 70% (vol/vol). The mixture was again centrifuged at 7,000 rpm for 15 min. The supernatant was discarded, and the pellet was resuspended in 500 μl of lysis buffer. Each sample was divided into two portions, and the nucleic acids were precipitated with cold ethanol. Chromosomal DNA was collected with a pipette tip and discarded. Samples were then centrifuged at 14,000 rpm for 25 min, and the pellets were washed with 70% ethanol (vol/vol). One portion from each

sample was treated by the addition of NaIO_4 to 40 mM in a final volume of 500 μl and incubated at 0°C for 90 min; this treatment inactivates uncharged tRNA. The reaction was stopped with the addition of rhamnose to a final concentration of 330 mM, and the solution was incubated at 0°C for 30 min. The samples were precipitated with ethanol and centrifuged at 14,000 rpm for 20 min. The pellet was washed with 70% (vol/vol) ethanol, resuspended in 500 μl of 1 M lysine, and incubated at 45°C for 1 h. The samples were precipitated with ethanol and centrifuged at 14,000 rpm for 20 min, and the pellets were resuspended in 50 μl of lysis buffer. The samples (6 μg nucleic acid per well based on A_{260}) were loaded, fractionated by gel electrophoresis in 8% (wt/vol) polyacrylamide-8 M urea, and subjected to Northern blotting analysis as described previously (4). Probes used for Northern blots are described in Table S2 in the supplemental material. The percentage of tRNA that was charged was calculated as the ratio of intensities of slow- and fast-migrating species.

RESULTS

mRNA ratios. All mRNA abundance ratios determined in this study and their *P* values are tabulated in Table S1 in the supplemental material. Differential mRNA levels were generally regarded as significant if *P* values were less than 10^{-5} . Consistent trends across operons were also considered significant at higher *P* values. mRNA abundance ratios are plotted in Fig. 1 and 2. Figure 1 illustrates our approach to the assessment of nutrient limitation effects, in which two comparisons are made for each nutrient limitation. For example, Fig. 1A shows that the mRNA ratios for both the leucine limitation versus the phosphate limitation and the leucine limitation versus the H_2 limitation are used to generate a plot in which mRNA levels affected by leucine limitation are along the diagonal. Similarly, Fig. 1B and C show the effects of phosphate limitation and H_2 limitation, respectively. Figure 2 presents a plot in which mRNA levels affected similarly by growth rate both when H_2 is limiting and when phosphate is limiting are along the diagonal. In each plot, colors are used to indicate other significant effects, so that genes whose mRNA levels are affected by more than one condition can be discerned. In addition, mRNA ratios for selected genes are given in Table 1. Certain ratios were confirmed by real-time RT-PCR, which showed similar trends (Table 1 and reference 14).

(i) Leucine limitation. mRNA levels affected by leucine limitation cluster along the diagonal plot shown in Fig. 1A. Leucine limitation resulted in increased mRNA levels for nearly all ribosomal proteins, as well as for certain translation elongation factors and subunits of RNA polymerase. These genes included Mmp0259 to Mmp0260, Mmp0640 to Mmp0641, Mmp0667 to Mmp0669, Mmp1319 to Mmp1325, Mmp1360 to Mmp1371, Mmp1403 to Mmp1421, and Mmp1543 to Mmp1547 and are exemplified by Mmp0259 and Mmp1409 (Table 1). mRNA differences were moderate but consistent, ranging to threefold. Levels of rRNA showed similar trends, with values for 16S rRNA ($\mu\text{g}/\text{OD}_{660}/\text{ml}$ of culture) of 11.8 ± 1.8 for leucine limitation, 9.0 ± 2.5 for phosphate limitation, and 7.4 ± 1.8 for H_2 limitation. The same set of RNAs was also affected by growth rate (see below). Leucine limitation also increased mRNA levels for two large gene clusters (Mmp0414 to Mmp0430 and Mmp1335 to Mmp1364) that appear to encode diverse functions.

Remarkably, leucine limitation had only modest effects on mRNA for amino acid biosynthetic functions. Of special importance, the levels of mRNA for both of the genes encoding the acetohydroxy acid synthase (*ilvB* and *ilvN*, Mmp0650 to

FIG. 1. Scatter plot of mRNA ratios for nutrient limitations. (A) Leucine limitation. $\log_2$ mRNA ratios for leucine limitation/phosphate limitation (L/P) are plotted against $\log_2$ mRNA ratios for leucine limitation/ H_2 limitation (L/H). Circles indicate the open reading frames (ORFs) significantly affected by leucine limitation (ORFs where the magnitudes of both $\log_2$ ratios are at least 0.58 [twofold] and both P values are 10^{-5} or lower). Effects of other nutrients are also indicated: mRNAs whose levels were significantly affected by phosphate limitation (green), H_2 limitation (red), and both H_2 and phosphate (blue). (B) Phosphate limitation. $\log_2$ mRNA ratios for phosphate limitation/leucine limitation (P/L) are plotted against $\log_2$ mRNA ratios for phosphate limitation/ H_2 limitation (P/H). Circles indicate those ORFs that were significantly affected by phosphate limitation. Effects of other nutrients are also indicated: leucine limitation (brown), H_2 limitation (red), and both leucine and H_2 (blue). (C) H_2 limitation. $\log_2$ mRNA ratios for H_2 limitation/leucine limitation (H/L) are plotted against $\log_2$ mRNA ratios for H_2 limitation/phosphate limitation (H/P). Circles indicate those ORFs significantly affected by H_2 limitation. Effects of other nutrients are also indicated: leucine limitation (brown), phosphate limitation (green), and both leucine and phosphate (blue). RP, ribosomal protein; HP, hypothetical protein.

FIG. 2. Scatter plot of mRNA ratios for growth rates. $\log_2$ mRNA ratios for rapid growth/slow growth under H_2 limitation (H) are plotted against $\log_2$ mRNA ratios for rapid growth/slow growth under phosphate limitation (P). Circles indicate significant effects of growth rate under both nutrient conditions (ORFs where the magnitudes of both $\log_2$ ratios are at least 0.58 [twofold] and both P values are 10^{-5} or lower). Effects of limiting nutrients are also indicated: mRNAs whose levels were significantly affected by leucine limitation (brown), phosphate limitation (green), and H_2 limitation (red). Where more than one nutrient had an effect, colors are such that leucine takes precedence over phosphate, which takes precedence over H_2 . RP, ribosomal protein; HP, hypothetical protein; Fd, ferredoxin.

Mmp0651), the first step in branched-chain amino acid biosynthesis, increased by 20 to 55% in leucine-limited versus in phosphate-limited or H_2 -limited cultures (Table 1). Although increases were small, these increases were statistically very significant. In contrast, the mRNA levels for *leuB* (Mmp0539), which is specific for leucine biosynthesis, was not affected similarly. Somewhat larger increases in mRNA levels were also found for a few genes in histidine and arginine biosynthesis (Mmp0013, Mmp0417, Mmp0947, and Mmp1013).

Leucine limitation had modest negative effects on mRNA levels for most genes of methanogenesis and early steps in carbon dioxide assimilation. For instance, leucine limitation modestly decreased mRNA levels for the AMP-forming acetyl-coenzyme A synthetase (AcsA, Mmp0148), the tungsten-containing formylmethanofuran dehydrogenase (Fwd, Mmp1244 to Mmp1249), the pyruvate oxidoreductase (Por, Mmp1502 to Mmp1507), and the methylreductase (Mcr, Mmp1555 to Mmp1559) (Table 1). mRNA abundance for some genes of methanogenesis also increased during H_2 limitation (14), and the leucine effect was reflected in the H_2 -versus-leucine ratios, which were larger than the H_2 -versus-phosphate ratios.

(C) H_2 limitation. $\log_2$ mRNA ratios for H_2 limitation/leucine limitation (H/L) are plotted against $\log_2$ mRNA ratios for H_2 limitation/phosphate limitation (H/P). Circles indicate those ORFs significantly affected by H_2 limitation. Effects of other nutrients are also indicated: leucine limitation (brown), phosphate limitation (green), and both leucine and phosphate (blue). RP, ribosomal protein; HP, hypothetical protein.

TABLE 1. Expression ratios of selected genes^a

Biosynthesis activities	Open reading frame, gene, or enzyme	Leu/H ₂ ratio	<i>P</i> value	Leu/P _i ratio	<i>P</i> value	H ₂ /P _i ratio	<i>P</i> value	Rapid/slow growth ratio (under H ₂ limitation)	<i>P</i> value	Rapid/slow growth ratio (under P _i limitation)	<i>P</i> value
Ribosomal proteins	lsuL10E (Mmp0259)	0.83 (0.69)	2.7E-05	0.68 (0.54)	1.6E-07	-0.02 (-0.25)	6.0E-01	0.39 (0.97)	1.9E-04	0.21 (0.70)	1.7E-02
	lsuL14P (Mmp1409)	0.88	8.6E-06	0.52	1.1E-04	-0.10	1.2E-01	0.61	9.1E-08	0.49	1.2E-04
Amino acid biosynthesis	<i>ilvB</i> (Mmp0650)	0.45	1.2E-07	0.24	8.2E-08	-0.12	2.0E-02	0.10	1.2E-01	-0.20	4.6E-05
	<i>ilvN</i> (Mmp0651)	0.63	1.5E-09	0.34	3.4E-09	-0.15	2.0E-02	0.28	1.2E-03	-0.25	1.4E-05
	<i>leuB</i> (Mmp0539)	0.22	4.4E-03	-0.03	6.4E-01	-0.10	5.3E-02	0.18	4.2E-03	0.05	2.3E-01
	<i>argH</i> (Mmp0013)	0.22	3.2E-05	0.19	3.0E-06	0.03	7.1E-01	0.07	1.6E-01	0.06	5.6E-01
	<i>hisA</i> (Mmp0417)	2.1	3.8E-07	2.1	4.3E-06	-0.21	5.1E-02	0.55	2.2E-06	0.49	3.9E-07
	<i>hisG</i> (Mmp0947)	0.48	1.8E-07	0.34	3.5E-06	-0.11	1.6E-02	0.27	2.2E-04	0.31	1.2E-05
	<i>carB</i> (Mmp1013)	0.55	1.3E-06	0.42	2.4E-06	-0.23	7.7E-06	0.15	3.9E-05	-0.31	2.0E-02
Methanogenesis and carbon assimilation	Acetyl coenzyme A synthetase, AMP forming (<i>acsA</i> , Mmp0148)	-1.22	5.1E-06	-0.72	2.7E-08	0.57	6.0E-04	-0.70	3.1E-03	-0.03	9.4E-01
	Tungsten-containing formylmethanofuran dehydrogenase (<i>fwdD</i> , Mmp1247) ^b	-0.55	1.2E-04	-0.51	9.5E-07	0.12	3.0E-01	-0.30	1.8E-04	-0.08	1.6E-01
	Pyruvate oxidoreductase (<i>porE</i> , Mmp1503) ^b	-0.61 (-0.57)	1.1E-05	-0.42 (-0.17)	9.6E-07	0.40 (0.07)	2.3E-07	0.64 (1.55)	2.5E-07	0.34 (1.81)	1.9E-01
	Methyl coenzyme M reductase (<i>mcrD</i> , Mmp1556) ^b	-0.67	1.6E-03	-0.79	3.9E-04	-0.29	6.1E-02	-0.12	7.8E-02	0.42	6.9E-05
Phosphate transport	Mmp1095 (phosphate binding protein)	-0.25	1.3E-03	-6.14	1.2E-13	-5.50	1.5E-14	-0.59	1.8E-05	1.13	8.9E-02
S-layer protein	Mmp0875	-0.54	3.3E-02	-0.38	1.4E-01	0.38	1.0E-06	2.41	4.4E-10	2.18	3.8E-5
A ₀ A ₁ ATPase	<i>atpE</i> (Mmp1041) ^b	-0.59	2.2E-09	-1.12	2.4E-12	-0.57	1.5E-08	0.76	1.1E-08	0.67	4.2E-06
Flagella	<i>flaE</i> (Mmp1671) ^b	-2.59	5.6E-10	-1.53	1.8E-04	1.20	2.7E-07	0.40	5.4E-02	-0.11	8.7E-01

^a All ratios are log₂. Ratios from real-time RT-PCR are shown in parentheses.

^b For proteins encoded by multiple genes, the values given are for the gene with the median leu/P_i value.

(ii) **Phosphate limitation.** Phosphate limitation increased mRNA levels for the phosphate ABC transporter (Mmp1095 to Mmp1098) (Fig. 1B and Table 1). Regulation was marked, ranging from 8-fold to 64-fold. Similarly, a phosphate ABC transporter was regulated at the transcriptional level by phosphate starvation in *E. coli* (30).

(iii) **H₂ limitation.** The effects of H₂ limitation are plotted in Fig. 1C. Specific effects on mRNA levels from the genes of methanogenesis and carbon assimilation were reported in reference 14.

(iv) **Growth rate.** Two growth rate comparisons were conducted, one where H₂ was the limiting nutrient and one where phosphate was the limiting nutrient. Specific growth rates that differed 4.5-fold to 4.8-fold were compared. Similar to leucine limitation, the mRNA levels for nearly all ribosomal proteins, as well as certain translation elongation factors and subunits of RNA polymerase, were increased by rapid growth (Fig. 2 and Table 1). mRNA level differences were moderate but consistent, ranging up to twofold. In most cases, growth rate effects were observed whether H₂ or phosphate was the limiting nutrient (for ribosomal protein operons Mmp1319 to Mmp1325 and Mmp1543 to Mmp1547, the growth rate effect under phosphate limitation was inconclusive due to data scatter). Levels of rRNA showed similar trends, with values for 16S rRNA (μg/OD₆₆₀/ml culture) for rapid and slow growth under H₂ and phosphate limitation of 11.6 ± 0.6 versus 6.2 ± 2.2 and 9.2 ± 1.0 versus 5.2 ± 2.0, respectively.

mRNA levels for one of two genes encoding S-layer proteins (SlpB, Mmp0875) increased fivefold with rapid growth rate (Fig. 2 and Table 1). S-layer proteins form a coat on the outer surface of *M. maripaludis* and those of many other archaea and bacteria. *M. maripaludis* carries a second S-layer protein, Slp, but this gene product was not regulated by growth rate, even though the protein is evidently more abundant (33).

(v) **Complex effects on ATPase and flagellum synthesis.** Two gene clusters exhibited complex trends. mRNA levels for the A₀A₁ ATPase (Mmp1038 to Mmp1047, e.g., Mmp1041) (Table 1) were highest under phosphate limitation, intermediate under H₂ limitation, and lowest under leucine limitation conditions; leucine limitation had the greatest effect, decreasing mRNA levels (Fig. 1A). Rapid growth had a modest but consistent stimulatory effect (Fig. 2). Effects on flagellar genes (Mmp1666 to Mmp1676, e.g., Mmp1671) (Table 1) were also complex, with leucine limitation having a negative effect and H₂ limitation having a positive effect (Fig. 1A and C). These results were consistent with observations for *Methanocaldococcus jannaschii*, where in batch culture, H₂ limitation increased flagellum synthesis (21). Growth rate effects were also complex, though moderate in magnitude: rapid growth increased flagellar mRNA when H₂ was limiting but decreased mRNA when phosphate was limiting (Fig. 2). These observations show that complex regulatory patterns could be elucidated with a multifaceted study based on array analyses of samples from continuous culture.

TABLE 2. Intracellular amino acid levels under nutrient-limiting conditions

Amino acid	mRNA level (nmol/mg [dry wt]) ^a			
	Batch culture ($\pm$ SD)	H ₂ -limited conditions	P-limited conditions	Leu-limited conditions
Thr	5.03 $\pm$ 0.04	2.24	9.56	10.75
Ser	0.92 $\pm$ 0.02	2.70	3.18	3.55
Pro	4.05 $\pm$ 0.05	13.86	11.67	9.63
Gly	2.40 $\pm$ 0.03	25.70	11.47	9.07
Ala	2.27 $\pm$ 0.03	5.40	6.51	5.14
Val	2.72 $\pm$ 0.04	1.23	5.29	15.51
Met	0.05 $\pm$ 0.01	0.05	0.05	0.41
Ile	0.47 $\pm$ 0.00	1.54	5.78	16.61
Leu	0.44 $\pm$ 0.00	1.49	3.80	0.22
Tyr	0.50 $\pm$ 0.00	0.73	2.20	3.01
Phe	0.48 $\pm$ 0.03	0.60	0.74	1.58
Lys	9.64 $\pm$ 0.07	5.71	17.62	12.96
His	0.60 $\pm$ 0.01	0.19	0.51	0.41
Arg	1.07 $\pm$ 0.02	0.78	4.48	8.89
Glu	223.84 $\pm$ 2.77	291.21	475.92	383.99
Asp	48.76 $\pm$ 0.05	27.14	114.27	46.62
Total	303.23 $\pm$ 5.59	380.58	673.04	528.37

^a The values for batch culture are the means $\pm$ standard deviations for three samples from the same culture. For each nutrient-limited condition, cell pellets from two replicate chemostat cultures were combined.

Cellular free amino acid pools. Cellular levels of free amino acids were measured for continuous cultures from each nutrient limitation. In addition, as a control for reproducibility, three separate samples from a batch culture were analyzed. Results from the batch culture samples (Table 2) showed that the analyses were highly reproducible. The high levels of glutamate under all growth conditions were consistent with its role as a compatible solute in these marine organisms. In a comparison of the three nutrient-limited conditions in continuous culture, H₂ limitation had general effects, while the effects of leucine limitation were more specific (Table 2). Thus, H₂ limitation resulted in an overall decrease in cellular amino acid levels compared to levels under phosphate limitation and leucine limitation conditions. A notable exception was glycine, which increased more than twofold with H₂ limitation. Leucine limitation resulted in markedly decreased leucine levels as expected. The other two branched-chain amino acids, valine and isoleucine, increased about threefold in leucine-limited cells compared with those in phosphate-limited cells, suggesting that there is regulation of branched-chain amino acid synthesis. Leucine limitation resulted in other changes as well, compared to phosphate limitation: methionine increased substantially, tyrosine, phenylalanine, and arginine increased moderately, and lysine and aspartic acid decreased moderately. The reasons for these last changes are not understood.

tRNA charging. The percentage of charging was measured for many of the tRNAs in H₂-, leucine-, and phosphate-limited cultures (Table 3). In general, the fractions of tRNAs that were aminoacylated (charged) were similar in the continuous cultures. In the H₂-limited culture, although the pool sizes of most free amino acids dropped more than twofold, the charging levels of most tRNAs decreased by less than 10%. The similar charging levels regardless of the variations in amino acid pool sizes suggest that the supplies of free amino acids were high

TABLE 3. Percentage of aminoacylated tRNA under nutrient-limiting conditions^a

tRNA	Anticodon	Codon(s) decoded	% under H ₂ -limited conditions	% under leucine- limited conditions	% under phosphate- limited conditions
F	GAA	UUU, UUC	(76)	85	86
Y	GUA	UAU, UAC	85	85	88
W	CCA	UGG	(67)	88	89
K	UUU	AAA, AAG	76	66	73
R1	GCG	CGU, CGC	77	77	84
R2	UCG	CGA, CGG	(75)	89	89
R3	UCU	AGA, AGG	16	15	12
P1	GCG	CCC, CCU	26	23	(27)
P2	UGG	CCA, CCG	79	77	75
C	GCA	UGU, UGC	(69)	67	71
L1	UAA	UUA, UUG	62	39	84
L2	GAG	CUC, CUU	(53)	41	67
L3	UAG	CUA, CUG	58	40	65
V1	GAC	GUC, GUU	51	84	84
V2	UAC	GUA, GUG	53	16	20
H	GUG	CAU, CAC	61	61	64

^a Values in parentheses indicate that the signal was faint.

enough to saturate most tRNAs, even in the H₂-limited culture. Studies of tRNA charging in *E. coli* showed that the charged fractions of all tRNAs are at about 80% during exponential growth (8, 16, 37). In *M. maripaludis*, most tRNAs were charged at levels of more than 60%; however, several individual tRNA isoacceptors, e.g., R3 and P1, had charging levels of less than 30%.

In leucine-limited cells, the charging levels of tRNA^{Leu} significantly decreased, reflecting the leucine limitation of the culture. This trend held for all three tRNA^{Leu} isoacceptors. This phenomenon is consistent with the decreased tRNA charging levels in *E. coli* strains starved for arginine, threonine, or leucine (29).

Leucine limitation also caused a shift in the relative charging of the three tRNA^{Leu} isoacceptors L1, L2, and L3. Based on the genomic sequence, codons read by L1, L2, and L3 are present in the proportions 1:0.4:0.05. This codon usage was reflected by the higher fraction of charged L1 isoacceptors than L2 and L3 in H₂- and phosphate-limited cells. However, with leucine limitation, the charged level of L1 decreased most dramatically and reached the same low level as those of L2 and L3. In general, the charged levels of tRNA isoacceptors are expected to change differently under amino acid starvation conditions depending on the concentrations of isoacceptors and codon usage (11). The low level of charged L1 may limit protein synthesis generally, while the relatively moderate changes in the charged levels of L2 and L3 may make the expression of certain proteins, which prefer to use rare codons, less sensitive to leucine starvation.

DISCUSSION

Leucine limitation. Both pathway-specific and general regulation of amino acid biosynthesis has been observed for hydrogenotrophic methanogens. In *Methanothermobacter marburgensis*, enzyme activities for tryptophan and leucine biosynthesis were specifically elevated during starvation for

each respective amino acid, suggesting specific regulation. However, the presence of leucine also decreased tryptophan as well as leucine biosynthetic enzyme activities, as did other amino acids, suggesting that general regulation also occurs. At the mRNA level, the *trp* operon expression was strongly increased under conditions of starvation for tryptophan, suggesting specific regulation, but it also increased during leucine starvation, suggesting general regulation as well (12). Recently the specific mechanism of the *trp* regulation by tryptophan has been reported in the related archaeon *Methanothermobacter thermautotrophicus* (34). In *Methanococcus voltae*, the transcription of *hisA* was specifically activated by starvation for histidine (27). However, mutants resistant to the histidine analog 1,2,4-triazole-3-alanine excreted histidine, proline, phenylalanine, and tyrosine, suggesting that the biosynthesis of not only histidine but also unrelated amino acids was increased (28). These results indicate that specific and general controls of amino acid biosynthesis are present in methanogens, but the extent and molecular mechanisms are still unknown.

To determine the response of *M. maripaludis* to leucine limitation, we subjected cultures of a leucine auxotroph to leucine-limited growth in continuous culture and compared the results to those of cultures limited by H_2 or phosphate. Two observations confirmed that the cells were leucine limited: charging levels of $tRNA^{Leu}$ were markedly and specifically decreased and the cellular leucine pool was decreased. Measurements of cellular amino acid pools showed that a regulatory response occurred in the branched-chain amino acid pathway, since valine and isoleucine levels increased. The threefold change in valine and isoleucine compared to the modest 20 to 55% change in mRNA levels for acetohydroxy acid synthase, a key regulated enzyme in this pathway, suggests that a combination of transcriptional and posttranscriptional regulation had occurred. Effects on the mRNA levels of other amino acid biosynthetic genes occurred as well. Their small magnitude as well as their occurrence in unrelated amino acid pathways are consistent with a general control of amino acid biosynthesis. (It should be noted that the chemostat cultures contained high levels of all the amino acids, except for leucine, a situation that would be expected to depress the effect of leucine limitation on transcription.) Hence, under leucine limitation conditions with *M. maripaludis*, there appears to be a specific effect on branched-chain amino acid biosynthesis at the enzyme level but a general effect on amino acid synthesis at the mRNA level. The specific inhibition of branched-chain amino acid biosynthesis at the enzyme level is consistent with the known properties of acetohydroxy acid synthase in *M. maripaludis* strain JJ, which is related to strain S2 used in the present study. This enzyme is partially inhibited by isoleucine and valine (K_i values in the range of 0.1 to 0.2 mM) and more completely inhibited by leucine (a K_i value of 1.8 mM) (36). Assuming that cells contain 2.5 μ l of water per mg (dry weight) (10), the intracellular concentrations of leucine were 1.5 mM in phosphate-limited cells and 0.1 mM in leucine-limited cells, a range that would result in higher activity leading to higher levels of isoleucine and valine during leucine limitation. Feedback inhibition of acetohydroxy acid synthase by one or more of the branched-chain amino acids is well known in *E. coli* and other organisms (25). On the other hand, the lack of specific regulation of branched-chain amino acid synthesis at the transcrip-

tional level is unusual. In this regard, it is interesting to note that *M. maripaludis* lacks an operon-based organization for many of its biosynthetic pathway genes, including those for branched-chain amino acids, that would facilitate transcriptional regulation by specific amino acids (15). Taken together, these observations suggest that *M. maripaludis* uses a strategy where the transcription of the amino acid biosynthetic genes responds to general indicators of the availability of amino acids and not the availability of specific amino acids.

While pathway-specific regulation was absent, a general increase in mRNA levels for ribosomal proteins occurred with leucine limitation, resembling the response of "relaxed" strains of *E. coli* which lack a stringent response (6, 20). In archaea, both relaxed and stringent strains have been observed (3, 5, 26). In addition, leucine limitation decreased mRNA levels for genes of methanogenesis. This effect may reflect a general strategy for conserving resources when amino acid availability limits growth.

Phosphate limitation. In contrast to leucine limitation, the cellular response to phosphate limitation was highly specific to a few operons. This result demonstrates that specific transcriptional responses in fact occur in this organism and contrasts with the more general response to leucine limitation.

H_2 limitation. The effects of H_2 limitation on mRNA levels for genes of methanogenesis and carbon dioxide assimilation have been reported (14). Levels of most of these mRNAs increased (markedly for steps involving the electron-carrying deazaflavin coenzyme F_{420}), presumably to adjust for the shortage of electrons required for these functions. In the current study, H_2 limitation generally decreased free amino acid pools. This result extends the observation that in *Methanococcus voltae* the enzymes of branched-chain amino acid biosynthesis are reduced three- to fivefold in specific activity by growth at low H_2 - CO_2 levels (35). These observations may reflect the energy demand that amino acid biosynthesis places on the cell. Like many methanogens, *M. maripaludis* can fix CO_2 as the sole source of carbon. However, autotrophic growth occurs at great expense in terms of energy and reducing equivalents, and we estimate that amino acid biosynthesis in *M. maripaludis* growing autotrophically accounts for about 45% of H_2 consumption. The incorporation of some exogenous amino acids occurs but can alleviate the H_2 demand only partially, since exogenous amino acids were incorporated into about 26% of the cellular carbon (32). Hence, the condition of limitation of H_2 , the electron donor for energy-yielding methanogenesis, presumably reduces the energy supply for amino acid synthesis. In addition, H_2 is the source of electrons for biosynthesis, and its limitation may directly affect crucial steps in amino acid synthesis pathways, such as biosynthesis of pyruvate.

Growth rate. The regulatory effects of growth rate have not been widely studied in archaea. Two genes stand out in the correlation of their mRNA levels with growth rate, one encoding an S-layer protein and the other encoding H_2 -dependent methylene tetrahydromethanopterin dehydrogenase (Hmd), a low-affinity hydrogenase that is one of two options for the reduction of methenyltetrahydromethanopterin to methylene tetrahydromethanopterin (14). These mRNA ratios approximated the differences in growth rate itself. Other effects of rapid growth, i.e., the increase in mRNA levels for translation

and transcription functions and ATPase and the effects on mRNA levels for flagellum synthesis, were more moderate.

We also observed that rapid growth resulted in an increase in the abundance of 16S rRNA as well as mRNA abundance for ribosomal proteins (and translation factors and RNA polymerase subunits encoded in the same operons). This is consistent with responses seen with *E. coli* and yeast (17, 18, 22). While similar studies with archaea have been limited, an increase in 16S rRNA abundance with rapid growth was seen in the archaeon *Pyrococcus furiosus* (7).

Concluding remarks. This work has extended our knowledge of global responses to nutrient limitations and growth rates in the *Archaea*. Many questions remain with respect to both the generality and the mechanisms of the responses. Leucine limitation, as well as growth rate, affected the synthesis of ribosomes, but how this occurs and whether the limitation for other amino acids will have a similar effect remain to be determined. The effects of leucine limitation on amino acid biosynthesis appeared to have both general and specific components, and it will be interesting to determine whether similar patterns arise from the limitation for other amino acids and organic precursors.

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