APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1993, p. 610–613
0099-2240/93/020610-04$02.00/0

Vol. 59, No. 2

Amino Acid Requirements of Two Hyperthermophilic Archaeal Isolates from Deep-Sea Vents, *Desulfurococcus* Strain SY and *Pyrococcus* Strain GB-D†

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Received 22 June 1992/Accepted 10 November 1992

Two sulfur-dependent hyperthermophilic archaea, *Desulfurococcus* strain SY and *Pyrococcus* strain GB-D, which were isolated from deep-sea hydrothermal vents, utilized free amino acids and peptides obtained from various molecular size fractions of yeast extract. It was found that 11 amino acids were essential for growth. The metabolic products were acetate, *i*-butyrate, and *i*-valerate.

Obligately heterotrophic, anaerobic, sulfur-dependent hyperthermophiles have been isolated from deep-sea hydrothermal vents (6, 9, 15, 16). It has generally been reported that the growth media suitable for these organisms appear to be limited to media containing complex proteinaceous substrates, such as yeast extract or tryptone, as sole carbon and energy sources. No growth is obtained on media containing simple carbohydrates, organic acids, or alcohols. In order to explain this metabolic behavior, we determined first which components of the complex proteinaceous substrates are utilized during growth and then whether certain amino acids are specifically required.

For this study we used two sulfur-dependent hyperthermophiles, *Pyrococcus* strain GB-D (20) and *Desulfurococcus* strain SY (9). Both of these strains were isolated from outer scrapings of black smoker chimneys; strain GB-D was isolated from the Guaymas Basin vent site (Gulf of California; depth, 2,020 m), and strain SY was isolated from 11°N on the East Pacific Rise (depth, 2,505 m). These organisms were grown in anaerobic tube cultures at 90°C as previously described (9), unless specified otherwise. Physiological characteristics of these organisms were recently described by Jannasch et al. (10).

The nutritional requirements of strains GB-D and SY were examined by using a series of organic substances as sole carbon and energy sources in a defined artificial seawater medium (ASW) (9) supplemented with 1% (wt/vol) sterilized elemental sulfur, 5 ml of Wolfe mineral elixir (22) per liter, and 5 ml of a solution containing 10 vitamins (1) per liter. Ammonium sulfate was used as the nitrogen source in ASW. The pH was set at 7.2. The media were flushed with N_2 and were reduced by adding approximately 400 μM sodium sulfide. Cell numbers were determined by acridine orange epifluorescense microscopy (2, 7).

Both organisms grew on media containing complex proteinaceous substrates, including yeast extract, peptone, tryptone, and polypeptone, but did not grow on media containing Casamino Acids, oligopeptides (including di-, tri-, tetra-, and pentapeptides), glucose, maltose, starch, lactate, pyruvate, formate, acetate, propionate, ethanol, methanol, glycine, alanine, serine, or histidine. While strain GB-D did not grow on media containing purified proteins, including casein, ovalbumin, thyroglobulin, bovine serum albumin, and gelatin, strain SY grew well on all of the media containing these compounds except the medium containing gelatin.

Next, we examined the patterns of utilization of various molecular weight fractions of yeast extract. Using Amicon ultrafiltration membranes (types YM5, YM2, and YC05), we separated a 10% filter-sterilized yeast extract solution into the following four molecular mass fractions: more than 5 kDa, 1 to 5 kDa, 0.5 to 1 kDa, and less than 0.5 kDa. The organisms were then grown on media containing each of these fractions (diluted 1:20 in ASW medium) as the sole carbon source; medium containing untreated yeast extract was used as a control. The two isolates grew at similar rates in all of the media containing the various molecular weight fractions of yeast extract (Fig. 1). The final cell yield

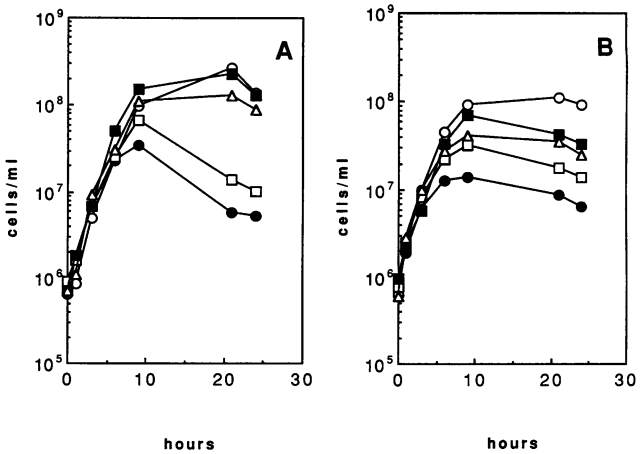

FIG. 1. Growth of *Pyrococcus* strain GB-D (A) and *Desulfurococcus* strain SY (B) on medium containing yeast extract and on media containing yeast extract molecular weight fractions (basal medium containing ASW, trace metals, vitamins, and elemental sulfur). Symbols: ○, medium containing complete yeast extract; ●, medium containing the more-than-5-kDa fraction; □, medium containing the 1- to 5-kDa fraction; ■, medium containing the 0.5- to 1-kDa fraction; △, medium containing the less-than-0.5-kDa fraction.

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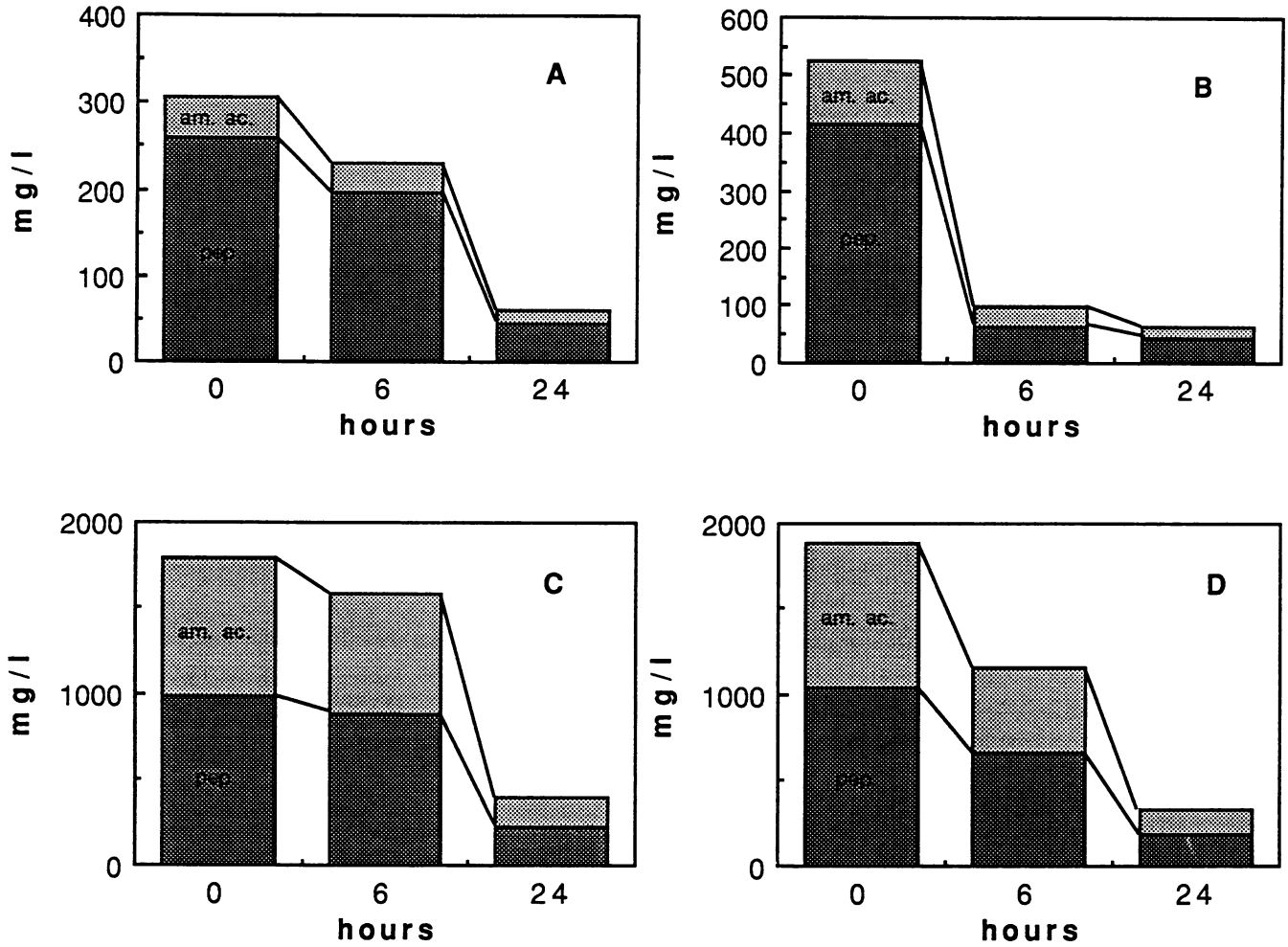

FIG. 2. Results of a time course experiment to determine the consumption of peptides and amino acids in medium containing the 1- to 5-kDa yeast extract fraction (A and B) and in medium containing the less-than-0.5-kDa yeast extract fraction (C and D) by *Pyrococcus* strain GB-D (A and C) and *Desulfurococcus* strain SY (B and D). The bars show the total amounts of peptides (pep.) and free amino acids (am. ac.) before growth (zero time) and after 6 and 24 h of growth in media containing the yeast extract fractions.

depended on the initial carbon content in each fraction. For example, untreated yeast extract contained 33% carbon, 67.7% of which was present in the 0.5- to 1-kDa fraction.

The compositions of free and hydrolyzable amino acids and their concentrations in two of the four fractions (1 to 5 kDa and less than 0.5 kDa) and untreated yeast extract were analyzed before (zero time), during (6 h), and after (24 h) growth of the organisms by using a Hitachi model L-8500 amino acid analyzer and following standard hydrolysis procedures. Surprisingly, both isolates consumed almost all of the amino acids of each fraction and of yeast extract by the end of the exponential growth phase. The consumption of asparagine, the consumption of glutamine, and the consumption of tryptophan were not clear because of difficulties in the determination of these compounds. The two strains consumed free amino acids and peptides in the untreated and fractionated yeast extract equally during growth. Similar patterns of consumption of amino acids and peptides were found with the 1- to 5-kDa and less-than-0.5-kDa fractions (Fig. 2).

The metabolic end products in spent 0.2% yeast extract medium with a cell density of 10^8 cells ml^{-1} were analyzed by using a Shimadzu model GC-14A gas chromatograph equipped with a Unisol F-200 30/60 3φX 6-m glass column (Gaskuro Kogyo Inc., Tokyo, Japan) and a flame ionization detector. In both strains the products were acetate (approximately 130 mg $liter^{-1}$), *i*-butyrate (approximately 40 mg $liter^{-1}$), and *i*-valerate (approximately 80 mg $liter^{-1}$).

Because of acid treatment during production (4), Casamino Acids (Difco) lack tryptophan, asparagine, and glutamine (14). Recently, Snowden et al. (18) reported that acid-hydrolyzed casein lacks cysteine, tryptophan, and glutamine but not asparagine. Neither of the strains grew on medium containing Casamino Acids (2 g $liter^{-1}$) alone, but both grew well when the medium was supplemented with tryptophan, asparagine, and glutamine (each at a concentration of 0.1 g $liter^{-1}$). By elimination, tryptophan was determined to be the essential amino acid of these three, and 50 μM tryptophan added to medium containing Casamino Acids was sufficient to support good growth of both isolates. In this respect *Pyrococcus* strain GB-D differs *Pyrococcus furiosus* (18), which did not grow on medium containing acid-hydrolyzed casein and the three missing amino acids.

In addition, medium containing a mixture of 20 individual

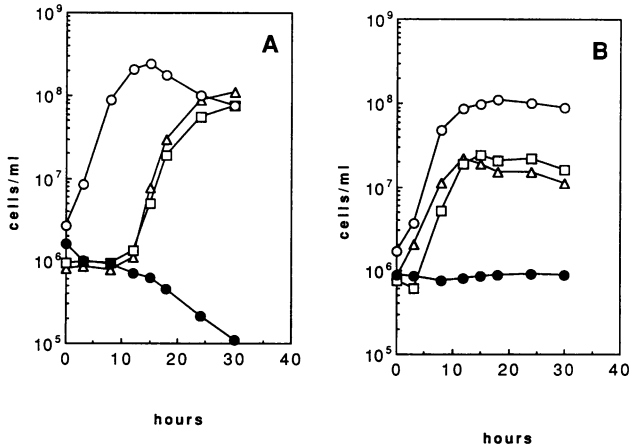

FIG. 3. Growth of *Pyrococcus* strain GB-D (A) and *Desulfurococcus* strain SY (B) on medium containing Casamino Acids, on medium containing Casamino Acids, tryptophan, asparagine, and glutamine, and on medium containing a mixture of 20 individual amino acids. Symbols: ○, medium containing yeast extract (2 g/liter); ●, medium containing Casamino Acids (2 g/liter); △, medium containing Casamino Acids (2 g/liter) plus tryptophan, asparagine, and glutamine (each at a concentration of 0.1 g/liter); □, ASW medium containing mixture of 20 amino acids (each at a concentration of 0.1 g/liter), trace metals, vitamins, and elemental sulfur (see text).

amino acids in ASW (glycine, alanine, serine, threonine, cysteine, asparagine, glutamine, leucine, isoleucine, valine, methionine, phenylalanine, tyrosine, tryptophan, proline, aspartic acid, glutamic acid, histidine, lysine, and arginine, each at a concentration of 0.1 g $liter^{-1}$), 1% (wt/vol) sulfur, vitamins, and trace elements supported the growth of both isolates (Fig. 3). In the amino acid-containing medium strain GB-D exhibited a longer lag than it exhibited in medium containing yeast extract, while strain SY exhibited no lag and had a lower final cell yield (2×10^7 to 3×10^7 versus 10^8 cells ml^{-1}). These phenomena may indicate that additional growth factors are present in complex proteinaceous substrates. In an additional series of growth studies, amino acids were deleted individually from the medium containing 20 amino acids described above. Our data revealed that threonine, leucine, isoleucine, valine, methionine, phenylalanine, tyrosine, histidine, lysine, and arginine were essential for growth of both isolates in addition to tryptophan. The lack of tryptophan in gelatin (19) may explain why neither of our isolates grows on this substrate. While the genera *Desulfurococcus* and *Pyrococcus* are classified in different kingdoms, the crenarchaeota and the euryarchaeota (21), the amino acid requirements of our two isolates appear to be similar. It is also interesting that the same amino acids (with the exception of tyrosine) are essential to animals (17).

A significant difference between the two isolates which we studied occurs in their ability to grow on medium containing proteins. While strain SY grows on medium containing purified proteins, strain GB-D does not. Preliminary studies revealed higher protease activities in the former strain, similar to the activities reported by Blumentals et al. (3) for *P. furiosus*.

Various growth responses to complex proteinaceous substrates have been reported for other hyperthermophilic archaea. *P. furiosus* utilizes Casamino Acids only with a low yield (5) or not at all (18). *Thermofilum pendens* (24) and *Hyperthermus butylicus* (25) can both grow on medium containing gelatin, but the latter cannot grow on medium containing a mixture of 20 amino acids or some synthesized peptides. These results suggest that the amino acid requirements of these organisms are different from those of strains GB-D and SY, which were obtained from deep-sea hot smoker sulfide deposits. In these high-temperature niches the occurrence of the proteinaceous substrates used for growth by the hyperthermophilic heterotrophs is most likely the result of anaerobic chemolithotrophic production by the hyperthermophilic methanogens isolated from these sites, including *Methanococcus* sp. (11, 12, 23) and *Methanopyrus* sp. (8, 13).

We thank S. J. Molyneaux, Y. Komamine, and Y. Tsuboi for excellent technical assistance, Y. Niimura, M. Kawamata, M. Shibayama, and N. Katakura for analyses of amino acids, dissolved organic carbon, and inorganic compounds, and S. Ito, T. Henmi, and E. Arai for analyses of specific amino acids. We also thank S. Kaneko, Y. Nakanishi, and S. Miyachi for support in this study.

This study was supported in part by National Science Foundation grant OCE89-00458 and by ONR grant N00014-91-J-1751.

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