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L-Lysine Production at 50°C by Mutants of a Newly Isolated and Characterized Methylotrophic *Bacillus* sp.

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The amino acid L-lysine was produced from homoserine auxotrophic and *S*-(2-aminoethyl)-L-cysteine-resistant mutants of a newly isolated gram-positive methylotrophic bacterium, capable of growth on methanol at 60°C. The temperature optimum for growth was between 50 and 53°C. These aerobic, gram-positive, endospore-forming, rod-shaped bacteria required biotin and vitamin B_{12} for growth. Extracts of the bacteria grown on methanol lacked hydroxypyruvate reductase and contained hexulose 6-phosphate synthase activity. Therefore, these bacteria were considered to be type I methylotrophic bacteria of the genus *Bacillus*. Fed-batch fermentations resulted in cell densities of 50 g of cell dry weight per liter. Biomass yields on carbon, nitrogen, phosphate, and sulfate were determined. Generation of homoserine auxotrophic and amino acid analog-resistant mutants resulted in L-lysine concentrations of nearly 20 g/liter in fed-batch fermentations.

Methylotrophs, microorganisms that use reduced one-carbon compounds, are diverse and ubiquitous (3, 20). Even though many methylotrophs have been described, only a few are gram positive (1, 2, 14, 21, 30). The industrial advantages of using methanol as a substrate for large-scale production of fermentation products have been outlined by Lentkin and Niekus (29). Methanol is relatively inexpensive, prices are relatively stable, production exceeds demand, and it is easily stored and transported. In addition, methanol is available in pure form, it is highly soluble in water, methanol solutions are not explosive, and residual methanol can be easily removed from products after fermentation is completed (29). *Bacillus* species have been used extensively in industrial fermentation processes, but little information has been published on the isolation of *Bacillus* species capable of rapid growth on methanol at temperatures above 50°C (2, 6, 14).

The nutritionally important amino acid L-lysine is a member of the aspartate family of amino acids and is primarily used in supplements for animal feeds made from grains that contain only limited quantities of L-lysine. Poultry, swine, and other livestock are unable to synthesize L-lysine and therefore must have this amino acid supplied as part of their diet. Currently, L-lysine is being manufactured by either direct fermentation (40; Tosaka et al., U.S. patent 4275157, June 1981; Miwa et al., U.S. patent 4560654, December 1985) or enzymatic conversion of DL-α-amino-ε-caprolactam (40). Fermentation processes that use strains of *Corynebacterium glutamicum* or *Brevibacterium lactofermentum* with molasses or starch hydrolysate as feedstocks have become the major method of L-lysine production (40).

This paper describes the isolation and characterization of a gram-positive, endospore-forming methylotroph that grew rapidly on methanol at 60°C and did not sporulate readily at temperatures above 50°C. Mutants of this methylotroph that are homoserine auxotrophs and *S*-(2-aminoethyl)-cysteine (AEC) resistant were capable of secreting significant amounts of the amino acid L-lysine.

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MATERIALS AND METHODS

Media. Minimal salts (MS) medium contained the following, in 1 liter of distilled water: K_2HPO_4, 3.8 g; $NaH_2PO_4 \cdot H_2O$, 2.8 g; $(NH_4)_2SO_4$, 3.6 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 2 mg; $CuSO_4 \cdot 5H_2O$, 40 μg; H_3BO_3, 30 μg; $MnSO_4 \cdot H_2O$, 200 μg; $ZnSO_4 \cdot 7H_2O$, 200 μg; $Na_2MoO_4 \cdot 2H_2O$, 47 μg; $CaCl_2 \cdot 2H_2O$, 5.3 mg; $CoCl_2 \cdot 6H_2O$, 40 μg. The pH was adjusted to 7.0 prior to autoclaving. Phosphates were reduced by half when MS medium was used for continuous cultures. The minimal vitamin (MV) medium was MS medium supplemented with thiamine hydrochloride, D-calcium pantothenate, riboflavin, and nicotinamide, each at 50 μg/liter; biotin and folic acid, each at 20 μg/liter; and vitamin B_{12} at 1 μg/liter. Yeast extract (MY) medium was MV medium supplemented with yeast extract, 0.5 g/liter. MV and MY contained 0.5% (vol/vol) methanol, unless otherwise stated. Other media used were nutrient broth and J medium (JV) prepared with added vitamins as in MV medium (17). Sporulation medium (SM) was composed of 3 parts nutrient broth and 4 parts MV medium. All solid media were prepared by combining double-strength medium components with an equal amount of 3% Bacto-Agar (Difco Laboratories, Detroit, Mich.) after autoclaving.

Enrichment isolation. Two 1-liter Omni-Culture fermentors (The VirTis Co., Gardiner, N.Y.) were used for enrichment by continuous culture. A peristaltic pump (S-280; Ismatec Mini, Chicago, Ill.) fed unsterilized MS medium into the vessels (0.1 to 0.5 h^{-1}), and a separate pump fed methanol at a rate that maintained a residual concentration of approximately 2 g/liter in the outflow. Methanol was measured by gas chromatography (GC). The pH was automatically controlled at 6.8 by the addition of 10% (vol/vol)

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ammonium hydroxide (Controller model 5656-00; Cole-Parmer Instrument Co., Chicago, Ill.). The temperature was maintained at 53 to 56°C with a circulating, temperature-controlled water bath coupled to the fermentor heat exchangers. A suspension of freshwater marsh soil, heated for 20 min at 90°C, was used as inoculum for the 1-liter batch fermentors operating at 53°C. When growth was evident in the vessels, the medium pumps were turned on and the dilution rate was gradually increased to produce continuous cultures for enrichment. Samples from the fermentors were periodically streaked on MV agar and incubated at 53°C. Isolated colonies obtained from these plates were restreaked and grown under the same conditions. The fastest growing isolates from the secondary plates were tested for growth on methanol by inoculating 2 ml of MV medium in 18-mm tubes with Bellco closures and incubating in a gyratory water bath at 53°C. Isolates that demonstrated growth in the MV broth were streaked onto MV agar for further purification. Gram stain, spore stain, and poly-β-hydroxybutyrate staining were done as described elsewhere (15). Gram stains were verified with the KOH test (18). Cell size was determined with cells grown for 18 h at 50°C on MY agar.

Characterization. The API Rapid CH and API Rapid E strip systems (Sheerwood Medical, Plainview, N.Y.) were used to provide a fermentation study of 49 substances and nine biochemical determinations. The test strips were inoculated and read according to the directions provided. Cultures used to inoculate two sets of strips were grown for 18 h at 55°C on JV and SM agar media instead of nutrient agar medium. Tests for nitrate reduction, NaCl tolerance, tyrosine decomposition, and lysozyme tolerance were performed as described before (17) but with the following changes. The reduction of nitrate to nitrite, NaCl tolerance, and lysozyme tolerance were tested in JV medium. Tryosine decomposition was tested in JV medium with tyrosine (5 g/liter) and 0.5% (vol/vol) methanol. Potassium nitrate (5 g/liter) was substituted for ammonium sulfate in MV medium to test the utilization of nitrate as a nitrogen source. Plates to detect hydrolytic activity were prepared by adding soluble starch (3 g/liter), fruit pectin (Certo brand; 10 g/liter), gelatin (Sigma type I; 4 g/liter), and casein (Carnation Non-Fat Dry Milk; 15 g/liter) to MV agar medium. Hydrolytic activities were detected as described elsewhere (28). Dipicolinic acid was extracted by autoclaving 5-ml samples of cell suspensions for 20 min. The samples were cooled, acidified with 1 ml of 1 N acetic acid, allowed to stand for 1 h, and centrifuged at 12,000 × *g* for 10 min. Dipicolinic acid content in the supernatant fractions was determined by a colormetric assay (24). Sporangia and cell counts were determined visually with the use of a Petroff-Hauser counting chamber. To determine heat resistance, aliquots of vegetative and spore-containing cultures were heated to 80°C for 10 min: viable and heat-stable counts were determined on MY agar plates after 48 h of incubation at 50°C. A spore suspension was prepared from a culture that had been grown in MY medium at 50°C for 6 h and then at 37°C for 18 h. The spore-containing culture was centrifuged at 12,000 × *g*, washed and suspended with distilled water, and pasteurized at 65°C for 10 min. Aliquots of this suspension were heated at 80°C for 10 min, and spore counts were determined on MV agar plates. To detect chloroform resistance, chloroform was mixed with aliquots (5 μl/ml) of culture and incubated at 37°C for 10 min prior to dilution and plating on MY agar. Antibiotic-containing disks (Difco) were used to test susceptibility. Cells used for enzyme assays were harvested in mid-exponential phase, suspended in 50 mM phosphate buffer (pH 7.0), and disrupted by two passages through a French pressure cell operated at 103 MPa. The cell debris was separated by centrifugation at 12,000 × *g*, and the supernatant was used as the crude extract. Hexulose phosphate synthase and hydroxypyruvate reductase were assayed as described previously (12, 27). Protein concentrations were determined with biuret reagent (10). The DNA base composition was determined by measuring the hyperchromic shift in absorbance as a function of temperature in 0.12 M sodium phosphate (pH 6.8), using *Escherichia coli* DNA as a standard.

Growth and cell yield. The growth response to various substrates was determined in MV medium by measuring the turbidity at 650 nm. The media contained alcohols at 0.5% (vol/vol) and sugars, organic acids, and methyl-substituted amines each at 0.3%. The effects of pH on growth were determined in MV medium with the pH initially adjusted by the addition of HCl or NaOH.

Cell dry weight (CDW) was determined by direct correlation with A_{500} as described previously (35), except that cells were grown in MV medium and washed with MV medium lacking methanol. CDW was determined from a plot of grams of cells per liter versus A_{500}, with an absorbance of $1.00 = 0.310 \pm 0.015$ g of dry cells per liter.

Cell yields on nutrients were determined at 50°C in baffled 500-ml Erlenmeyer flasks, rotated at 300 rpm, containing 100 ml of MV medium with various amounts of the limiting nutrient. Growth was followed by reading the turbidity each hour at 500 nm. Yields were determined from a plot of final CDW versus initial nutrient concentration. For determination of yields on phosphate, 60 mM 3-(*N*-morpholine)propanesulfonic acid (MOPS) buffer was used in place of phosphate in the MV medium. When the yield on sulfate was determined, all metal sulfates were replaced by their chlorides and ammonium chloride was used in place of ammonium sulfate.

Lysine determination and shake flask screen for lysine producers. Lysine was determined in culture supernatants by the acidic ninhydrin assay method (40). Amino acids were also quantitated by high-pressure liquid chromatography, using precolumn derivatization with *o*-phthalaldehyde and fluorescence detection of the *o*-phthalaldehyde amino acid derivative (25).

Potential lysine producers were cultured in medium that contained, per liter, 10 g of K_2HPO_4, 32 g of $(NH_4)_2SO_4$, 10 g of $CaCO_3$, 0.2 g of $MgCl_2 \cdot 6H_2O$, 20 g of methanol, trace metals and vitamins at the concentrations described for growth in the fermentor, and 200 mg of L-threonine and L-methionine per liter. Strains were cultured in 25 ml of medium in 250-ml baffled Erlenmeyer flasks covered with two 6-in. (15.24-cm) milk filter disks (nongauze; Kendall), and a piece (10 by 10 cm) of 2-mil Teflon membrane (Chem Fab Inc., West Palm Beach, Fla.) to reduce evaporation. The cultures were started by using a 2 to 5% inoculum and grown at 50°C in an air shaker with a revolution rate of 300 rpm. Samples were taken and the concentration of methanol was determined every 12 h by GC, using a Shimadzu GC with a Hayesep R (Alltech) column. The column temperature was 120°C and the detector temperature was 250°C. Methanol was added when the concentration dropped below 200 mM. Experiments were carried out for 24 to 48 h.

Auxotroph production and analog resistance. Cells to be mutagenized were grown to late log phase in MV medium containing 0.2% Casamino Acids. A 2.5-ml portion of culture was combined with 2.5 ml of fresh medium, and either 50 μl of ethyl methanesulfonate or 250 μg of *N*-methyl-*N'*-nitro-

N-nitrosoguanidine was added followed by shaking for 10 to 20 min. This was followed by dilution (25- to 50-fold) and growth in MV medium containing either 0.2 to 0.4% Casamino Acids or 50 μg of the amino acids of the desired auxotroph per ml, or both. After 6 h of growth, the culture was diluted with 3 volumes of methanol-free medium and incubated at 37°C for 18 h. The spores that formed were collected by centrifugation at 12,500 × *g*, washed twice with sterile water, and stored at 4°C. Dilutions of the spores were plated onto amino acid-containing plates, which were incubated at 50°C for 36 h. Colonies were replicated to amino acid-containing and minimal media, and incubation was overnight at 50°C. Auxotrophs were selected for specific amino acid requirements. Auxotrophs were confirmed by absolute growth requirements and growth response to amino acids added to MV medium.

Selection for resistance to AEC was achieved by plating mutagenized cells on MV medium containing 1 g of methionine per liter, 1 g of threonine per liter, and AEC. Cells were then plated on increasing levels of AEC until they would no longer grow. Single colonies were screened for lysine production in 18-mm culture tubes and in shake flasks. The best isolates were remutagenized, and this procedure was repeated until no further increase in lysine production could be observed. Several isolates were selected by screening mutagenized cells surviving for 8 h in MV medium containing lysine (1 g/liter), methionine and threonine (50 mg/liter), and the threonine analog β-hydroxynorvaline (HNV).

Fermentation conditions. Fed-batch cultivation of cells was carried out at 50°C in either a 14-liter (Chemap) or a 7-liter (New Brunswick) reactor, using 11 or 5 liters as the working volume, respectively. The medium contained, per liter, 3.09 g of K_2HPO_4, 0.9 g of $NaH_2PO_4 \cdot H_2O$, 2 g of $(NH_4)_2SO_4$, 0.2 g of $MgCl_2 \cdot 6H_2O$, 50 μg of biotin, 10 μg of vitamin B_{12}, 3.98 mg of $FeCl_2 \cdot 4H_2O$, 7.36 mg of $CaCl_2 \cdot 2H_2O$, 9.9 mg of $MnCl_2 \cdot 4H_2O$, 136 μg of $ZnCl_2$, 54.4 μg of $CuCl_2 \cdot 2H_2O$, 80.4 μg of $CoCl_2 \cdot 2H_2O$, 96.8 μg of $Na_2MoO_4 \cdot 2H_2O$, 59.6 μg of H_3BO_3, and 3.2 g of methanol. The agitation rate was 382 cm/s (920 rpm), and the aeration rate was 5.5 liters/min (0.5 vol/vol per min); the pH was maintained at 7.1 by the automatic addition of 8 N ammonium hydroxide. Phosphate, magnesium, and calcium levels were maintained by automatically feeding a solution of 100:10:1 phosphate-magnesium-calcium (1 M KH_2PO_4–0.1 M $MgCl_2$–0.01 M $CaCl_2$). Feeding of this nutrient mix was carried out by connecting the pump to the pH controller, so that the nutrient mix would be added whenever the ammonium hydroxide was added to adjust the pH. The pump speed was adjusted so that the rate of addition of the nutrient mix was twice the rate of ammonium hydroxide addition to maintain the desired optimal ratio of nitrogen, phosphate, magnesium, and calcium as determined by growth yields. Dissolved oxygen was monitored by using a galvanic probe and was maintained at 30% by oxygen-enriched aeration. Feeding of oxygen was monitored and controlled by a mass flow controller (Sierra Instruments Inc., Carmel Valley, Calif.) interfaced to a proportional controller (LFE Corp., Clinton, Mass.). Foaming was controlled with a liquid level controller (Cole-Parmer) by the automatic addition of a silicon-based anti-foam, SAG-471 (Union Carbide). Inlet and headspace gasses (carbon dioxide, oxygen, nitrogen, argon, methanol, ammonia, and water) were monitored by a Questor mass spectrometer interfaced to an IBM-AT computer. The inlet and headspace gasses were alternately sampled for 2 min (sample rate, 0.166 s^{-1}) after a 30-s delay to allow the line and ionization chamber to clear. The collected data were averaged every 5 min, stored on the computer, and used in calculation of oxygen uptake and carbon dioxide evolution rates. Methanol levels in the reactor were continuously monitored by using an in situ methanol sensor consisting of a silicone tubing (0.062-in. inside diameter by 0.095-in. outside diameter) probe connected to the flame ionization detector of a GC (44). One meter of silicone tubing was used in the probe with an air flow rate of 60 ml/min through the probe. The tubing connecting the probe to the GC was heated to 50°C with self-regulating heating tape, and the GC detector temperature was 250°C. The 0 to 5-V integrator output signal from the flame ionization detector of the GC was used to monitor and to operate the methanol feed pump (Watson-Marlow) automatically by use of a proportional controller (LFE Corp.) to maintain a methanol concentration of 100 mM in the reactor. A load cell was used to determine the amount of methanol that was fed to the reactor. The methanol also contained the required trace metals in the following concentrations: 1.09 g of $FeSO_4 \cdot 7H_2O$, 0.39 g of $MnCl_2 \cdot 4H_2O$, 22 mg of $ZnSO_4 \cdot 7H_2O$, 19 mg of $CoCl_2 \cdot 6H_2O$, 19 mg of $Na_2MoO_4 \cdot 2H_2O$, and 19 mg of $CuSO_4 \cdot 5H_2O$ per liter.

RESULTS

Enrichment and isolation. Development of a methanol-utilizing mixed culture at 55°C was rapid and abundant. Dilution rates were raised periodically to 0.45 h^{-1} without loss of the mixed culture. Smears revealed a preponderance of gram-positive forms, including endospore-forming bacteria, and a variety of morphological types, including some large pleomorphic cells. Bacteria that apparently grew on MV agar could be readily isolated from the enrichment vessels. However, only a rare isolate was found that grew rapidly when returned to liquid MV medium at 53°C, and this methanol-utilizing isolate was given the strain designation MGA3 (ATCC 53907). Additional strains were isolated from a batch enrichment of bog muck, using the same isolation techniques. These strains included strain NOA2, which had properties very similar to those of MGA3.

Cells and endospores. Cells of strain MGA3 were rod shaped (0.8 to 1.0 by 2.5 to 4.5 μm) with rounded ends. Early exponential-phase cultures stained Gram positive, and all cultures were KOH negative. Cells were motile but motility was usually not conspicuous. Colonies produced on MV agar were colorless, translucent, circular, and convex and had entire margins. Spores were oval and 0.8 to 1.0 μm by 1.1 to 1.2 μm, their location was subterminal, and sporangia were swollen. Supplemented agar media (MY and SM) produced more endospores than MV agar medium.

Endospores were not produced in MY broth at 50°C, but cultures grown at 50°C and shifted to 37°C during exponential phase contained 2.7 × 10^7 CFU/ml at 26 h and 47% of these counts were chloroform resistant. Refractile endospores were visible in the culture after 8 h at 37°C, and 50% of the cells contained endospores at 26 h. Dipicolinic acid was absent from vegetative bacteria but present in bacterial endospores. Cultures of MGA3 grown in MV medium at 50°C and then switched to 37°C had a dipicolinic acid content of about 3% (dry weight). Spore suspensions produced 7.4 × 10^7 CFU/ml on a methanol-salts minimal medium (MV) and 3.5 × 10^7 CFU/ml after heating at 80°C for 10 min.

Growth and growth substrates. Strain MGA3 grew poorly in nutrient broth or on nutrient agar. The isolate grew well in J medium, a complex medium used for the growth of fastidious species of *Bacillus* (17). The isolate grew rapidly

TABLE 1. Biomass yields for MGA3 on the major nutrients

Nutrient	Yield (g of CDW per g of nutrient)[a]
Methanol	0.48
Ammonium sulfate	1.1
Potassium phosphate (K_2HPO_4)	6.9
Potassium sulfate	38.5
Magnesium chloride	121
Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	720[b]
Manganese chloride ($MnCl_2 \cdot 4H_2O$)	2,000[b]

[a] Yields were determined in shake flasks at 50°C and confirmed in a 14-liter reactor.
[b] Determined only in a 14-liter reactor at 50°C.

when MV medium contained methanol or mannitol as a source of carbon and energy and grew slowly when it contained glucose. Maltose, ribose, acetate, glutamate, and α-ketoglutarate were utilized poorly. Galactose, lactose, sucrose, xylose, formate, succinate, glycerol, ethanol, *n*-propanol, *n*-butanol, methylamine, dimethylamine, and trimethylamine were not utilized.

Acid was produced from only 7 of the 49 substrates used in the API Rapid CH test (5-ketogluconate, D-glucose, D-tagatose, D-arabitol, mannitol, maltose, and ribose). Gas was not produced from any of these substrates. Strain MGA3 did not produce acid or gas from any of the following substrates: adonitol, amygdalin, arbutin, cellobiose, D-arabinose, D-xylose, D-fructose, D-mannose, dulcitol, D-raffinose, D-turanose, D-lyxose, D-fucose, erythrithol, esculin, glycerol, galactose, glycogen, gluconate, inositol, indulin, L-arabinose, L-xylose, L-sorbose, lactose, L-fucose, L-arabitol, melibiose, melezitose, *N*-acetylglucosamine, rhamnose, sorbitol, salicin, saccharose, starch, β-methylxyloside, trehalose, β-gentiobiose, xylitol, α-methyl-D-mannoside, α-methyl-D-gluconate, and 2-ketoglucanate.

Of the eight vitamin components in MV medium, biotin and vitamin B_{12} were required for growth. Nitrate was not utilized as a nitrogen source. Biomass yields from methanol, ammonia, phosphate, sulfate, magnesium, manganese, and iron are shown in Table 1.

Biochemical characterization. Crude cell extracts of MGA3 cultures grown on MV medium lacked hydroxypyruvate reductase activity but contained high hexulose 6-phosphate synthase activity. The specific activity of hexulose 6-phosphate synthase was 3.72 to 6.27 mmol of formaldehyde/min per mg of protein.

The API Rapid E tests indicated the presence of cytochrome oxidase and urease and the production of acetoin, while the tests for β-galactosidase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, phenylalanine deamination, and indole production were negative.

Strain MGA3 did not produce catalase or tyrosine-degrading enzymes. The isolate grew in JV broth that contained 1% but not 5% NaCl. Nitrate was not reduced to nitrite. Starch, gelatin, and pectin were hydrolyzed but growth was inhibited on casein-containing plates.

Strain MGA3 was susceptible to gentamicin, sulfadiazine, tetracycline, ampicillin, rifampin, chloromycetin, erythromycin, and penicillin G.

DNA isolated from strain MGA3 had a base content of 44 mol% G+C.

Morphological variant. Strain Gr was isolated from a culture of MGA3 grown on MY agar and is presumed to be a morphological variant of strain MGA3. The cells of strain Gr were large coccus shaped and therefore distinctly different from the typical rod shape of MGA3. Strain Gr shared all other characteristics, such as growth on methanol and mannitol and a requirement for biotin and vitamin B_{12}, with strain MGA3. Strain Gr also had a temperature optimum near 50°C, was susceptible to penicillin, and stained Gram positive; crude cell extracts of Gr contained levels of hexulose 6-phosphate synthase activity similar to those found in MGA3. Strain Gr, like strain MGA3, only formed significant quantities of heat-resistant endospores when an actively growing culture at 50°C was switched to 37°C for 18 h. Only methanol or mannitol supported the rapid growth of Gr.

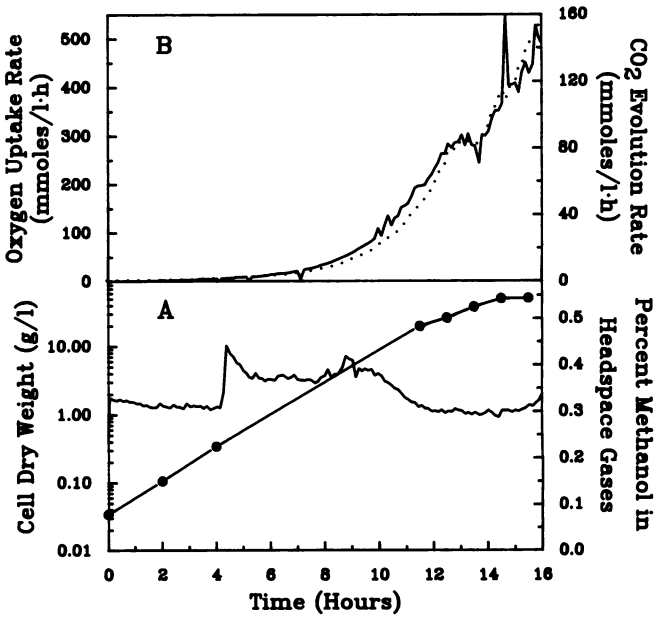

FIG. 1. CDW (●) and percent methanol in the headspace gases (—) (A) and oxygen uptake (—) and carbon dioxide evolution (●) rates (B) during fed-batch cultivation of MGA3. The growth rate during exponential phase was 0.48 h^{-1}, and the final CDW was 50 g/liters.

Growth on methanol. Growth of MGA3 in MV medium containing methanol was optimal near pH 7.0, and growth did not occur at pH 5.5. The optimum temperature for growth was found to be 50 to 53°C, with growth occurring from 37 to 60°C. At 37°C the growth rate was equal to 0.5 μ_{max} at 50°C. Growth of MGA3 in MY medium in the 14-liter reactor at 50°C resulted in a maximum growth rate of 0.8 h^{-1}, while growth on MV medium resulted in a maximum growth rate of 0.48 h^{-1}. Fed-batch fermentations, using an MS medium, resulted in cell densities as high as 50 g of CDW per liter in the 14-liter reactor. The cells grew exponentially to 50 g of CDW per liter with a growth rate 0.48 h^{-1} (Fig. 1), and the yield of biomass from methanol remained unchanged (0.48 g of CDW per g of methanol) from that determined in shake flasks. The methanol level in the reactor remained relatively constant throughout the fermentation as determined by both the methanol probe and the mass spectrometer. The oxygen uptake and carbon dioxide evolution rates indicated that growth was exponential throughout the first 14 h of the fermentation (Fig. 1).

Isolation of L-lysine producers. Homoserine auxotrophs were isolated from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-mutagenized cells of the thermophilic methylotroph, strain NOA2. Auxotrophy was confirmed in liquid cultures by demonstrating that either homoserine or threonine and me-

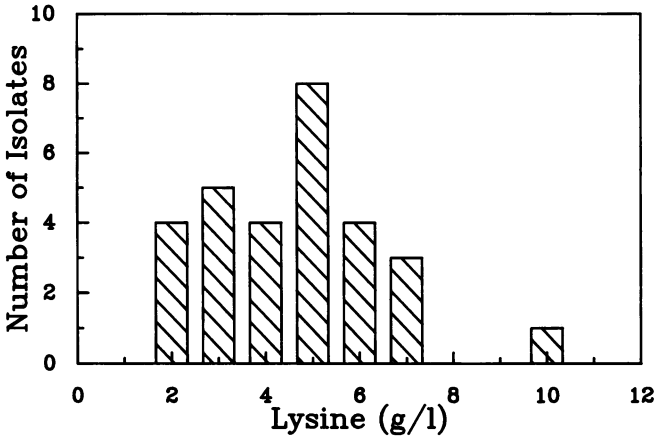

FIG. 2. Distribution of AEC-resistant mutants derived from strain NOA2 HNV#7 with respect to lysine production.

thionine were required for growth. Resistance to AEC was derived by plating mutagenized cells of the homoserine auxotrophs on plates containing 600 μg of AEC per ml. This resulted in the selection of isolates that produced L-lysine in the range of 0.5 g/liter. These isolates were screened for lysine production, and the best producer was used for further experiments. The isolate, designated NOA2 8/16-5, produced about 1 g of lysine per liter in a shake flask, but growth inhibition due to lysine at concentrations of >5 g/liter was observed in shake flasks. This inhibition was relieved by plating ethyl methanesulfonate-mutagenized cells on plates containing 20 g of L-lysine per liter and selecting the isolates that grew rapidly. Two of these isolates produced 2.6 to 2.8 g of lysine per liter in shake flasks. It was observed that L-threonine at levels of 100 mg/liter completely inhibited lysine formation. To overcome this inhibition, isolates were selected by using the threonine analog HNV. This resulted in the selection of one isolate that produced 7.8 g of lysine per liter in the shake flask, but the inhibition of lysine formation by threonine had not been completely eliminated. The screening of AEC-resistant mutants resulted in the distribution of lysine production shown in Fig. 2. Lysine-producing strains have also been developed from MGA3 and Gr, using the same techniques that were successful with NOA2. In fact, an auxotrophic strain of Gr produced an equivalent amount of lysine in a shake flask assay as NOA2 HNV#3.

L-Lysine production. Growth of the homoserine auxotrophs in the 14-liter reactor required the feeding of both threonine and methionine. This was accomplished by adding 17.6 g of L-threonine and 8.8 g of L-methionine per liter to the phosphate-magnesium-calcium feed solution. When the production of lysine in the shake flasks was found to be non-growth associated, the media were designed to limit growth by either phosphate or sulfate depletion. The pump feeding the amino acid-magnesium-calcium solution was stopped after growth of the cells had ceased, and the methanol feed was changed from the methanol-trace metals mix to pure methanol. This prevented the toxic effects caused by high concentrations of several of the trace metals. Fed-batch fermentations of NOA2 L20#1 HNV#3, using either phosphate or sulfate limitation to obtain 5 g of CDW per liter, resulted in formation of 8.2 and 7.8 g of L-lysine per liter, respectively. Figure 3 shows the non-growth-associated formation of lysine under phosphate-limited conditions in the 7-liter reactor.

Analysis of the culture supernatants from this fed-batch fermentation by high-pressure liquid chromatography revealed that aspartic acid also accumulated in the reactor (Fig. 3). The aspartic acid appeared only after lysine accumulation had begun and reached final levels that were between 30 and 60% of the final lysine concentration. No difference in the levels of aspartic acid were found when either phosphate or sulfate limitation was used. Production of lysine at cell densities of 20 g/liter, using phosphate limitation, resulted in L-lysine concentrations of 19 g/liter and aspartate concentrations of 7.0 g/liter after 32 h of cultivation (Fig. 4).

DISCUSSION

From the characteristics of strain MGA3 and the morphological variant Gr, these bacteria were assigned to the genus *Bacillus* (17). The appearance of Gr was similar to the *rod* mutants isolated from cultures of *Bacillus subtilis* and *B.*

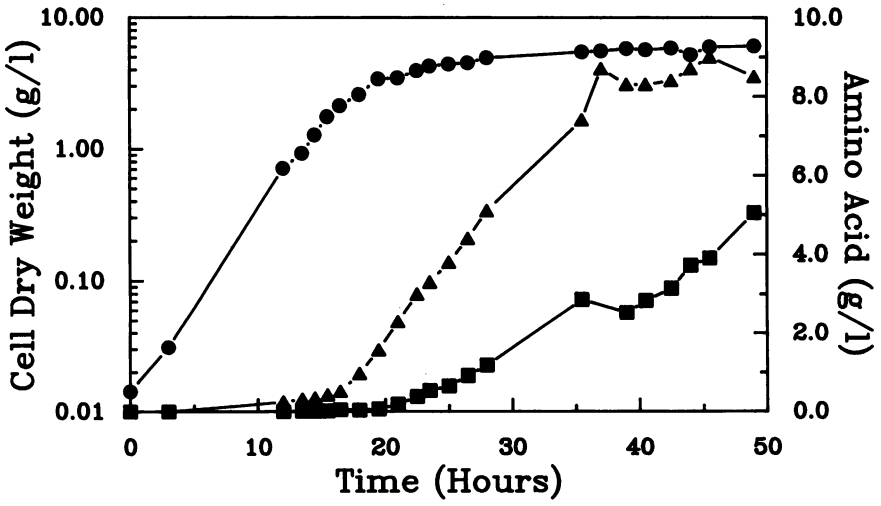

FIG. 3. Fed-batch fermentation of NOA2 L20#1 HNV#3 under phosphate limitation in the 7-liter reactor showing CDW (●), lysine (▲), and aspartic acid (■).

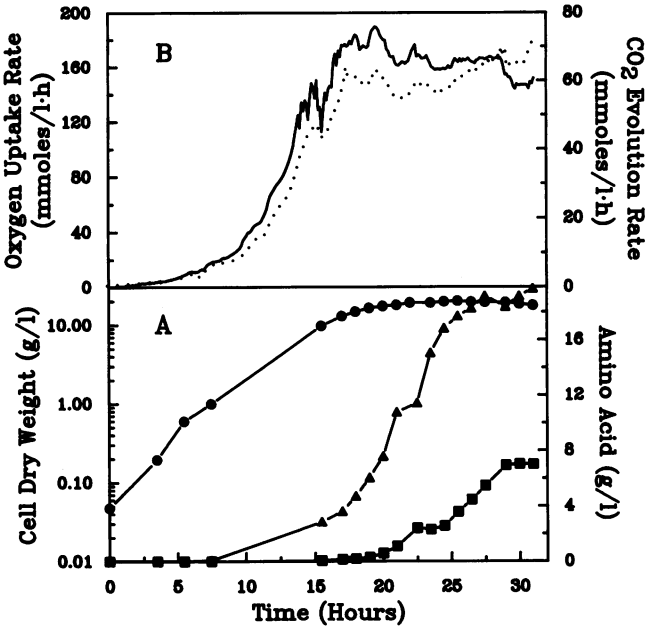

FIG. 4. Fed-batch fermentation of NOA2 L20#1 HNV#3. (A) CDW (●), lysine (▲), and aspartic acid (■). (B) Oxygen uptake (—) and carbon dioxide evolution (•) rates during the fermentation as determined by mass spectroscopy.

licheniformis by Rodgers et al. (34). In an attempt to define better a species classification for MGA3, the characteristics were compared with other *Bacillus* species. Many similarities to *B. stearothermophilus*, including cellular dimensions, spore morphology, lack of catalase, and others, were found. A major difference between MGA3 and *B. stearothermophilus* is the optimum growth temperature; 65 to 75°C for most *B. stearothermophilus* isolates and 50 to 53°C for MGA3. However, several strains of *B. stearothermophilus* belonging to group 2 (42) have been isolated that show a reduced optimum growth temperature (<70°C). Another major difference between MGA3 and *B. stearothermophilus* is that MGA3 can utilize methanol as the sole carbon and energy source. Even though a strain of *B. stearothermophilus* has been shown to contain an alcohol dehydrogenase capable of oxidizing methanol, this strain was unable to utilize methanol as a carbon source for growth (16). In view of these major differences between MGA3 and *B. stearothermophilus*, our isolate will be referred to in this report as *Bacillus* sp. strain MGA3.

Until recently the isolation of thermophilic, gram-positive, endospore-forming methylotrophs has been very difficult (2, 6, 14). Dijkhuizen et al. (14) have reported the isolation of several strains of gram-positive, endospore-forming, thermotolerant bacteria which they also designated as *Bacillus* spp. Brooke et al. (6) have reported a biomass yield of 0.5 ± 0.02 g of CDW per g of methanol from these strains grown in continuous culture. This biomass yield is essentially identical to the biomass yield of 0.48 g of CDW per g of methanol observed for our isolates. In another report (2), organisms that could utilize a large number of carbon sources including methylated amines and solvents such as isopropanol, isobutanol, and acetone were isolated from sewage sludge, and all but one of these isolates were assigned to the genus *Bacillus*. However, the ability to utilize a wide variety of carbon sources distinguishes these isolates from those isolated by Dijkhuizen et al. (14) and those described in this paper.

Designation of MGA3 as a type I methylotrophic bacterium resulted from its ability to utilize methanol as a carbon and energy source, the ability of resting cells and cell extracts to oxidize methanol, and the presence of the enzyme hexulose 6-phosphate synthase. MGA3 lacked hydroxypyruvate reductase, an enzyme found in type II methylotrophs (3). In addition, the organisms isolated by Dijkhuizen et al. (14) were also shown to be type I methylotrophs, while those isolated by Al-Awadhi et al. (2) were not classified.

There are significant advantages in using thermophilic organisms and thermophilic methylotrophs for industrial processes (11, 29, 36, 37). Two of these advantages, reduced risk of contamination and reduced consumption of cooling water, become very important in large-volume, high-cell-density processes. Fed-batch fermentations that achieve high cell densities, 40 to 250 g of CDW per liter, resulting in greater productivity, have been reported for a large number of bacteria and yeasts (26, 32, 39, 45). In fed-batch culture, the methanol-utilizing *Pseudomonas* sp. strain K (39) and *Protaminobacter ruber* (45) have been reported to reach cell densities of 160 and 85 g of CDW per liter, respectively, but neither of these organisms was thermophilic.

Development of an automated high-cell-density process for growth of the thermophilic methylotrophs reported here required the feeding of both phosphate and trace metals, since high levels of phosphate in the reactor caused the precipitation of metal phosphates during base addition. This may be due to the lower solubility of some metal phosphates at elevated temperatures. To overcome this problem, the metals and the phosphate feeds were separated by dissolving the trace metals in the methanol and using a separate feed reservoir for the phosphate. The trace metals were then fed coupled to the carbon source, while the phosphate feed was coupled to the ammonium (nitrogen) hydroxide source. Methods for calculating the metal ion concentration in the mineral feed and coupling the mineral feeding to either the carbon or nitrogen source have been developed previously (38). These feeding strategies were effective for cultivation of MGA3 to high cell densities in an automated fed-batch culture.

Amino acid production from methanol has not been reported previously due to the difficulty in obtaining auxotrophic mutants from obligate gram-negative methylotrophs (23, 31, 33). Bohanon et al. (5) were able to isolate stable tryptophan auxotrophs in *Methylophilus methylotrophus* AS1 by site-specific mutagenesis of the *trpE* gene. In contrast, amino acid auxotrophs were readily isolated, using the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or ethyl methanesulfonate, from *Bacillus* sp. strain MGA3, Gr, or NOA2.

For overproduction of L-lysine, both deregulation of aspartokinase and formation of a homoserine auxotroph are desired (40). In wild-type *B. megaterium*, sulfate limitation has also been shown to result in lysine excretion (9); however, neither MGA3, Gr, nor NOA2 excreted lysine under conditions of phosphate or sulfate limitation. Deregulation of aspartokinase has previously been accomplished in *Corynebacterium glutamicum* and *Brevibacterium flavum* by selecting for resistance to the L-lysine analog AEC (41), which has also been used successfully in *B. brevis* (22) and *B. licheniformis* (19). Isolation of a homoserine auxotroph from strain NOA2 and development of AEC resistance resulted in lysine overproduction and excretion into the medium (Table 2).

Evaluation of NOA2 8/16-5 L20#1 HNV#3 in the fermen-

TABLE 2. Characteristics of the L-lysine-producing mutants

Strain	Markers[a]	Lysine (g/liter)[b]
NOA2		None[c]
NOA2 8/16-5	Hse$^-$ AEC^r	1.0
NOA2 L20#1	Hse$^-$ AEC^r Lys^r	2.8
NOA2 HNV#3	Hse$^-$ AEC^r Lys^r	7.8
NOA2 AEC#27	Hse$^-$ AEC^r Lys^r	10

[a] Hse, Homoserine; Lys, lysine.
[b] Determined in shake flask cultures after 36 h of incubation at 50°C.
[c] Less than 1 mg/liter.

tor indicated that lysine formation was not associated with growth (Fig. 3), similar to the lysine-excreting strains of *B. megaterium* (7), *Micrococcus luteus* (8), and a homoserine auxotroph of *B. sphaericus* (4). Inhibition of lysine formation by threonine may be responsible for the non-growth-associated lysine formation by the homoserine auxotroph of *B. sphaericus* (4). Threonine inhibition of lysine formation was also observed in NOA2 8/16-5 L20#1 and is probably due to threonine repression or inhibition of aspartokinase. Even though the threonine antagonist HNV did improve lysine production (Table 2), it did not completely eliminate inhibition of lysine formation by threonine. In addition, the observed production of aspartic acid probably results from feedback inhibition or repression of aspartokinase by lysine, since the aspartic acid appears only after lysine has begun to accumulate (Fig. 3 and 4).

Lysine concentrations of 20 to 75 g/liter have been reported from a large variety of organisms (13, 40, 41), but all of these organisms are mesophiles. A lysine-overproducing strain of *B. licheniformis* was capable of growth at temperatures of >45°C with a temperature optimum for lysine production of 40°C (19). This organism, however, sporulated when grown at 40°C, and this may have prevented further lysine accumulation. To prevent sporulation, tetracycline was added to the medium (19). Lysine production from the thermophilic methylotrophs reported here did not encounter these problems because sporulation only occurred when the temperature was shifted from 50 to 37°C. A lysine concentration of nearly 20 g/liter, the higher temperature (50°C) at which lysine was produced, the ability to readily generate amino acid auxotrophs, and the ability to cultivate these organisms to high cell densities without sporulation in an automated fed-batch reactor indicate that these methylotrophic bacilli may be useful for amino acid production from methanol.

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