Single, Chemically Defined Sporulation Medium for *Bacillus subtilis*: Growth, Sporulation, and Extracellular Protease Production

JAMES H. HAGEMAN,* GARY W. SHANKWEILER, PAMELA R. WALL, KAREN FRANICH, GLORIA W. MCCOWAN, SUSAN M. CAUBLE, JESUS GRAJEDA, AND CASILDA QUINONES

Department of Chemistry, New Mexico State University, Las Cruces, New Mexico 88003

Received 12 March 1984/Accepted 6 July 1984

The composition and application of a single, chemically defined medium for growth and sporulation of *Bacillus subtilis* is described. At 37°C cells grew with a doubling time of about 40 min; cultures attained near-maximal spore formation (70 to 80% by 12 h after the end of exponential growth and produced 1×10^9 to 2×10^9 heat-resistant free spores at 24 h. Dipicolinic acid production was completed between 7 and 11 h. Cells grown in the single, chemically defined medium excreted levels of serine and neutral proteases comparable to those excreted in nutrient broth medium.

A single, chemically defined medium which would allow both rapid growth and synchronous, high-percentage sporulation of *Bacillus subtilis* would be useful in certain metabolic studies; studying of nutritional auxotrophs, labeling of cellular components with radioisotopes, and determining the effects of metabolites on cellular metabolism might all be more definitively done with a fully defined medium. The medium most widely used for studying sporulation in *B. subtilis* is the supplemented nutrient broth of Hanson et al. (10); however, it is not possible to answer certain questions using complex medium. For example, one cannot easily determine in such a medium whether amino acids, peptides, or proteins induce or repress production of proteases.

Donnellan et al. (6) developed the first single, synthetic medium for *B. subtilis* but reported maximal spore yields after 4 days; Mandelstam and Waites (12) have used this synthetic medium (omitting glucose) as a resuspension medium and have reported that cells resuspended in it sporulate to a high degree within 8 h. It has also been used to prepare spores (4), but little further description of this medium has appeared.

A semidefined medium which has been widely used is that devised by Sterlini and Mandelstam (17); cells are grown in a medium containing casein hydrolysate and transferred to a medium containing glutamate and salts to achieve sporulation. Using this medium, Doering and Bott (5) found that a tryptophan auxotroph of *B. subtilis* would not sporulate when placed in resuspension medium which lacked tryptophan. On the other hand, they found that a leucine auxotroph required no leucine in the resuspension medium to achieve normal levels of sporulation. These results raised the possibility that the Casamino Acids present in the growth medium might be affecting metabolic pathways essential for sporulation.

Ramaley and Burden (13) developed a set of chemically defined media in which they carried out growth and sporulation of *B. subtilis* using a resuspension technique. Cells were grown in a medium containing glucose and glutamate or ammonium lactate and inorganic salts, centrifuged, and resuspended in a medium containing glutamate or lactate and inorganic salts. Nearly complete sporulation was obtained within 8 to 10 h after resuspension. As with the Sterlini-Mandelstam procedure (17), this procedure has the

disadvantage of requiring two sets of media and the transfer of cells from one medium to another at the end of the growth phase. We found that when our standard strain (15), *B. subtilis* 168 (*trp*), and a low-protease strain derived from it were grown in Ramaley-Burden medium (13) both sporulated equally well (60 to 70% refractile bodies in 20 h) regardless of whether tryptophan was present or absent in the resuspension medium. This was in contrast to the observation of Doering and Bott (5) cited above and emphasized to us the dramatic effect of having casein hydrolysate in the growth medium on subsequent requirements of the cells. Bond et al. (2) have also recently reported striking effects of amino acids on rates of degradation of intracellular proteins. Consequently, we attempted to prepare the simplest possible, chemically defined, single medium which would allow growth and sporulation of *B. subtilis*.

Freese et al. (7) reported that a series of purine auxotrophs of *B. subtilis* grew and sporulated well in a chemically defined, morpholinepropanesulfonic acid (MOPS)-buffered medium, S-6, containing 0.2% glucose. We began the studies reported here by using MOPS-buffered S-7 medium (18) supplemented with 50 µg/ml each of the L-forms of the amino acids glutamate, tryptophan, isoleucine, leucine, methionine, arginine, proline, threonine, lysine, and phenylalanine. We systematically varied the levels of MOPS, glutamate, lactate, combinations of amino acids, sodium bicarbonate, ammonium sulfate, trace metals, pH, and the methods of inoculation to try to find the simplest medium and conditions which would give good growth, fairly synchronous sporulation, and high yields of spores. Ruppen and Switzer (14) have used a MOPS-buffered medium supplemented with 0.1% Casamino Acids to study the stringent response in *B. subtilis*, but they have not reported the ability of this medium to support sporulation.

Figure 1 shows growth and sporulation of *B. subtilis* 168 (*trp*) under the best conditions found to date; this defined sporulation medium is referred to hereafter as CDSM. Good sporulation could also be obtained when either glutamate or lactate was omitted, but our standard strain underwent less lysis and less clumping when both were present. For example, cultures of cells grown in 5 mM L-lactate in the absence of L-glutamate underwent a 36% decline in turbidity at 5 h compared with the turbidity at maximal growth. The kinetics of appearance of dipicolinic acid of cells growing in CDSM (Fig. 1) were closely similar to that previously

* Corresponding author.

FIG. 1. Growth, sporulation, and acid production of *B. subtilis* 168 (*trp*) in CDSM. Colonies were maintained on gelatin plates as previously described (9). An inoculum was made by transferring a single colony into 50 ml of CDSM in a 300-ml triple-baffled sidearm flask (Bellco Glass, Inc., Vineland, N.J.) which was shaken at 220 rpm in a G-25 incubator shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 37°C until turbidity reached 50 to 200 Klett units, usually in 10 to 12 h. Turbidity was measured on a Klett-Summerson colorimeter fitted with a no. 66 filter (Klett Manufacturing, New York, N.Y.). Cultures were inoculated by transferring a 2 to 5% (vol/vol) inoculum, depending on its turbidity, to 70 ml of prewarmed CDSM in a 300-ml triple-baffled flask and continuing growth under the same conditions as described above for the inoculum. CDSM was made by combining appropriate volumes of stock solutions, each adjusted to pH 7.0 (23°C) with KOH: 1.00 M MOPS, 0.05 M KH_2PO_4 , 1.00 M $(\text{NH}_4)_2\text{SO}_4$, 1.00 M D-glucose, 0.50 M L-lactic acid, and 1.00 M L-glutamic acid. Solutions were autoclaved for storage except for MOPS which was passed through a filter (0.45- μm pore size; Millipore Corp., Bedford, Mass.). A 50 $\times$ stock trace metal mix, the MT mix of Vasantha and Freese (18), contained: 0.200 M MgCl_2 , 70.0 mM CaCl_2 , 5.00 mM MnCl_2 , 0.10 mM ZnCl_2 , 0.50 mM FeCl_3 , 0.20 mM thiamin-hydrochloride, and 2.00 mM HCl. The FeCl_3 was added last, which prevented precipitation, and the stock was passed through a sterile filter (0.45- μm pore size). CDSM was prepared by adding appropriate amounts of the above stocks to deionized water to achieve final concentrations of: 40 mM MOPS, 4.0 mM KH_2PO_4 , 9.5 mM $(\text{NH}_4)_2\text{SO}_4$, 5.0 mM L-lactic acid, 8.0 mM L-glutamic acid, 50 μg of L-tryptophan per ml, 50 μg of L-isoleucine per ml, 1 $\times$ MT mix, and 20 mM D-glucose. MT mix and glucose were added sterilely, immediately before inoculation, to all other components which had been autoclaved together. Amino acids were added before autoclaving by weighing out appropriate amounts of solid forms. Spore counts were made by heating 100 $\times$ dilutions of culture medium in minimal salts medium at 70°C for 30 min and plating appropriate dilutions on gelatin-agar plates (9). Numbers in parentheses are percentages of spores based on viable cell estimations made from dilutions of unheated culture samples taken at the same time. Measurements of pH were taken on 1-ml clarified samples of culture broth with a combination glass electrode and an Orion model 811 pH meter (Orion Research, Inc., Cambridge, Mass.). Dipicolinic acid (DPA) was measured in autoclaved cell suspensions by the spectrophotometric method of Janssen et al. (11). Data points are averages of four determinations.

reported (3) for *B. subtilis* growing in Bacto-Peptide medium. No further increases occurred after 11 h, which suggests that the sporulation process is highly synchronous. Spores were also released quite efficiently from the cells; at 15, 20, and 30 h the free spores comprised 50, 85, and 95% respectively, of the total refractile bodies observed with a phase-contrast microscope. Once spores had formed in CDSM, they did not appear to germinate even when shaking in the same medium was continued for 79 h. The pH changes in CDSM were similar to those seen in nutrient broth medium (4).

We repeatedly observed a plateau in heat-resistant spore counts between 10 and 13 h, followed by an increase in spore counts. We believe, from microscopic observations, that the delayed increase in spores (after 14 h) is due to the breaking up of chains of cells. That is, the earlier counts (10 to 13 h) are probably underestimations of the true number of spores because chains of cells containing more than one spore are only giving rise to a single colony. In some experiments, we

observed a decline in turbidity between 3 and 6 h which was confirmed to be lysis by microscopic observations and by the corresponding decline in the viable cell count observed in these cultures. Declines in turbidity appeared to be caused by removing cultures from the growth chamber for excessive periods of time; minimizing sampling times greatly reduced or eliminated this problem (Fig. 1; see Fig. 3).

The consumption of the three carbon sources by *B. subtilis* 168 cells during growth and sporulation in CDSM was monitored (Fig. 2). Using a defined growth medium containing 0.7 mM L-glutamate and 5.6 mM D-glucose, Bernlohr and Switzer (1) found a simultaneous disappearance of the two from the medium, with the apparent consumption of D-glucose (on a micromole per milliliter basis) being only about twice as fast as that of L-glutamate. They too observed a net production of L-glutamate, but only during the stationary phase. Increase in pH correlated with the consumption of lactic and glutamic acids between 6 and 9 h of growth (Fig. 2), but the initial decline in pH did not

FIG. 2. Concentrations of D-glucose (○), L-glutamic acid (Δ), and L-lactic acid (□) in CDSM during growth and sporulation of *B. subtilis* 168. Growth in CDSM and measurements of turbidity and pH were carried out as described in the legend to Fig. 1. Lactic acid and glucose were measured with lactate dehydrogenase and glucose oxidase (Sigma Kits and Technical Bulletins 826-UV and no. 510, respectively; Sigma Chemical Co., St. Louis, Mo.). Glutamic acid was measured enzymatically by the method of Bernlohr and Switzer (1). Values reported are averages of duplicate or triplicate determinations; standard deviations were less than 10% of the values plotted. All assays were performed on samples of clarified culture broth; pH (▲) was measured as described in the legend to Fig. 1.

correlate with increases in glutamic and lactic acid. The latter suggests that other acids must be released during the fall in pH.

Finally, we measured production of cell dry matter and extracellular proteases by *B. subtilis* 168 during growth and sporulation in CDSM (Fig. 3). In this study, no decline in cell dry matter occurred between 3 and 6 h. When the data in Fig. 3 were compared with data for the same strain grown in nutrient broth (9), we found that at 8 h the cells grown in CDSM produced 90% as much serine protease per cell (1.9 units of absorbance at 335 nm per h per ml of culture per 100 Klett units) as those grown in supplemented nutrient broth. Furthermore, the kinetics of serine protease production and the amounts of neutral protease excreted by cells were very similar in the two media (9). We conclude that the complex mixture of amino acids, peptides, and proteins present in nutrient broth is not required in the growth medium for substantial excretion of the two major extracellular proteases of *B. subtilis*. In contrast, Vasantha and Freese (18) found that when *B. subtilis* cells were induced to sporulate in a defined medium by the addition of decoyinine (a GMP synthetase inhibitor), the cells produced only about 6% as much total extracellular protease (serine plus neutral protease) per cell at 8 h as they did when they sporulated in a supplemented nutrient broth. The decoyinine-treated cells, however, sporulated in the presence of excess glucose, which may repress protease synthesis (8).

CDSM should be useful for studying metabolism during sporulation in cases in which radiotracers are required. Preliminary studies on protein degradation of *B. subtilis* cells sporulating in CDSM have shown that the rates of degradation of proteins labeled with [14 C]leucine are at least as high as those found in cells sporulating in nutrient broth (16).

FIG. 3. Increase in dry mass and extracellular protease production by *B. subtilis* 168 in CDSM. Cells (●) were grown in CDSM as described in the legend to Fig. 1. Dry mass was determined by removing 2-ml portions at the times indicated and collecting the cells on a tared 13-mm (diameter) Millipore filter (0.45- μ m pore size). Filters and cells were dried at 100°C to constant weight. Weights were determined on a Mettler M5 microgram balance. Protease assays were performed with azocasein as a substrate as previously described (9). The neutral protease was that portion of total activity insensitive to phenylmethylsulfonyl fluoride, whereas the serine protease was that insensitive to EDTA.

This work was supported by Public Health Service grant 5R01-GM19643 from the National Institute of General Medical Sciences and by grant SO6RR-08136 from the Minority Biomedical Research Support Program.

Thanks are due to N. Vasantha for useful comments on the manuscript.

LITERATURE CITED

- Bernlohr, D. A., and R. L. Switzer. 1983. Regulation of *Bacillus subtilis* glutamine phosphoribosylpyrophosphate amidotransferase inactivation in vivo. *J. Bacteriol.* 153:937-949.
- Bond, R. W., A. S. Field, and R. L. Switzer. 1983. Nutritional regulation of degradation of aspartate transcarbamylase and of bulk protein in exponentially growing *Bacillus subtilis* cells. *J. Bacteriol.* 153:253-258.
- Chasin, L. A., and J. Szulmajster. 1967. Biosynthesis of dipicolinic acid in *Bacillus subtilis*. *Biochem. Biophys. Res. Commun.* 29:648-654.
- Deutscher, M. P., and A. Kornberg. 1968. Biochemical studies of bacterial sporulation and germination. VIII. Patterns of enzyme development during growth and sporulation of *Bacillus subtilis*. *J. Biol. Chem.* 243:4653-4660.
- Doering, J. L., and K. F. Bott. 1972. Differential amino acid requirements for sporulation in *Bacillus subtilis*. *J. Bacteriol.* 112:345-355.
- Donnellan, J. E., Jr., E. H. Nags, and H. S. Levinson. 1964. Chemically defined, synthetic media for sporulation and for germination and growth of *Bacillus subtilis*. *J. Bacteriol.* 87:332-336.
- Freese, E., J. Heinze, T. Mitani, and E. B. Freese. 1978. Limitation of nucleotides induces sporulation, p. 277-285. In G. Chambliss and J. C. Vary (ed.), *Spoors VIII*. American Society

- for Microbiology, Washington, D.C.
8. Freese, E. B., N. Vasantha, and E. Freese. 1979. Induction of sporulation in developmental mutants of *Bacillus subtilis*. *Mol. Gen. Genet.* **170**:67-74.
 9. Geele, G., E. Garrett, and J. H. Hageman. 1975. Effect of benzenboronic acids on sporulation and on production of serine proteases in *Bacillus subtilis* cells, p. 391-396. In P. Gerhardt, R. N. Costilow, and H. L. Sadoff (ed.), *Spores VI*. American Society for Microbiology, Washington, D.C.
 10. Hanson, R. S., J. Blicharska, and J. Szulmajster. 1964. Relationship between the tricarboxylic acid cycle enzymes and sporulation in *Bacillus subtilis*. *Biochem. Biophys. Res. Commun.* **17**:1-7.
 11. Janssen, F. W., A. J. Lund, and L. E. Anderson. 1958. Colorimetric assay for dipicolinic acid in bacterial spores. *Science* **127**:26-27.
 12. Mandelstam, J., and W. M. Waites. 1968. Sporulation in *Bacillus subtilis*. The role of exoprotease. *Biochem. J.* **109**:793-801.
 13. Ramaley, R. F., and L. Burden. 1970. Replacement sporulation of *Bacillus subtilis* 168 in a chemically defined medium. *J. Bacteriol.* **101**:1-8.
 14. Ruppen, M. E., and R. L. Switzer. 1983. Involvement of the stringent response in degradation of glutamine phosphoribosylpyrophosphate amidotransferase in *Bacillus subtilis*. *J. Bacteriol.* **155**:56-63.
 15. Spizizen, J. 1958. Transformation of biochemically defined *Bacillus subtilis* by deoxyribonucleate. *Proc. Natl. Acad. Sci. U.S.A.* **94**:1072-1078.
 16. Spudich, J. A., and A. Kornberg. 1968. Biochemical studies of bacterial sporulation and germination. VII. Protein turnover during sporulation of *Bacillus subtilis*. *J. Biol. Chem.* **243**:4600-4605.
 17. Sterlini, J., and J. Mandelstam. 1969. Commitment to sporulation in *Bacillus subtilis* and its relationship to actinomycin resistance. *Biochem. J.* **113**:29-37.
 18. Vasantha, N., and E. Freese. 1980. Enzyme changes during *Bacillus subtilis* sporulation caused by deprivation of guanine nucleotides. *J. Bacteriol.* **144**:1119-1125.