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Preparation of Refractile Spores of *Clostridium thermosaccharolyticum* Involves a Solventogenic Phase

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Conversion of vegetative cells of *Clostridium thermosaccharolyticum* to refractile endospores was achieved by sequential transfer and dilution at each generation, with a final dilution into a sporulation medium that contained xylan supplemented with excess calcium. The subsequent growth was synchronous and resulted in elongated, solventogenic cells that were then shifted to 35°C to permit further differentiation without cell division. The synchronized cells grown in xylan medium supplemented with Ca gluconate produced total solvents that reached 9.63% (vol/vol). One hundred percent of these elongated solventogenic cells (4.84×10^9 cells per ml) entered the sporangial stage and continued to differentiate into refractile spores. Only cells sequentially transferred and diluted at a critical time of the growth cycle are synchronized, induced to elongate (≥fourfold), become highly solventogenic in the presence of excess calcium, and are converted to a homogeneous population of refractile spores.

In *Clostridium thermosaccharolyticum*, it was observed that only cells that initially elongated to a critical cell length (8.9 to 10.0 μm) continued to differentiate; cells which failed to elongate to the critical length also failed to show any sign of terminal swelling and did not continue through the sequential stages to become spores (4, 11, 22). It was further observed that although sporulation in this *Clostridium* species was under catabolite repression, continuous feeding with glucose reversed the repression and resulted in elongation of individual cells and formation of sporangia (10). In addition, the highest frequency of initiation of sporulation in *C. thermosaccharolyticum* was increased when cells were synchronized in glucose medium and then shifted to continuous cultures at 60 min (generation time [G] = 67 min in glucose medium) (9). The synchronized cells elongated and formed swollen heads; however, free refractile spores were never observed (9). Hoffmann et al. (8) obtained highly synchronized cultures of *C. thermosaccharolyticum* in glucose medium for three generations. This synchronization resulted in elongation of individual cells, generation times considerably shorter than those of asynchronous vegetative cells, formation of sporangia, and significant amounts of ethanol. Similar patterns of synchronous growth, morphological change, and solventogenesis were also reported in a continuous dilution culture of *C. thermosaccharolyticum* utilizing xylan as the sole source of carbon (15, 16). However, attempts to produce a homogeneous population of free refractile spores of *C. thermosaccharolyticum* have been unsuccessful by the reported investigations.

In this study, we developed culture conditions that permit complete differentiation of synchronized, elongated, solventogenic cells to 100% free refractile spores.

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MATERIALS AND METHODS

Organism and media. National Canners Association strain 3814 of *C. thermosaccharolyticum* was used in this study. Preparation and activation of pea broth stock cultures was carried out as previously described (15). The basal medium was the modified medium of Hsu and Ordal (10) to which 0.5% (wt/vol) of the carbon source was added. The pHs of all of the media were adjusted to 7.0 with 1 M NaOH prior to sterilization. All sporulation media were identical to the growth medium, except that calcium was added in the form of $CaCO_3$ or Ca gluconate at a concentration of 0.1 M.

Conditions for synchronization and sporulation. Cells were grown in batch cultures as previously described (15, 16), with xylan as the carbon source. Following one generation of growth (ca. 4 h), 1 ml of the culture was transferred to 10 ml of fresh xylan medium and again grown as a batch culture for one generation. The cells from this second culture (ca. 10^8 cells per ml) were diluted 100-fold by inoculation into a sporulation medium and subsequentially grown as a third batch culture in serial dilution bottles that contained 150 ml of medium overlaid with 40 ml of sterile mineral oil and were sealed with screw caps. The cultures were incubated in a rotary water bath shaker at 56°C and 150 rpm to ensure complete mixing of the substrate with the microorganism. Inoculations were made, and samples were obtained aseptically with sterile 1-ml pipets. The screw cap was removed with concomitant insertion of a gassing syringe needle. After sampling, the screw cap was replaced at the top of the bottle and the gassing needle was brought halfway out of the neck; after approximately 30 s, the needle was withdrawn as the screw cap was fitted into place and tightened. Growth was continued in this final culture until the population increased to approximately 10^9 cells per ml, and greater than 50% of the cells elongated four times. At this time (ca. 45 h), the temperature of the shaker bath was lowered to 35°C within 2 to 3 min and maintained for the remainder of the incubation period. Growth was measured throughout the incubation by monitoring the changes in optical density at 600 nm and by differential counting with a Petroff-Hausser counting chamber and a phase-contrast microscope at a magnification of ×400. All experiments were carried out in triplicate.

TABLE 1. Sporulation in *C. thermosaccharolyticum* following a temperature shift

Sporulation medium[a] (generation time [min])	Total cell count (10^9 cells/ml)	No. of refractile spores (10^9)	% Refractile spores
Xylan-$CaCO_3$ (195)	1.00	0.17	17.0
Xylan-Ca gluconate (157)	5.40	4.84	89.6
Ca gluconate (125)	1.10	0.03	2.7

[a] Calcium compounds were added to 0.1 M.

Determination of fermentation end products. Supernatant fluid from the culture samples was analyzed by gas-liquid chromatography and detected by flame ionization. Separation of primary metabolites was done as previously described (15). The percentage (vol/vol) of each metabolite produced was calculated on the basis of the area of standard reference peaks and converted to millimolar concentrations.

Ion chromatographic analysis of *C. thermosaccharolyticum*. Cells from the culture fluid without supplemented calcium were centrifuged in a small-angle centrifuge with an SP/X rotor at 2,200 × *g* (Sorvall, Inc., Norwalk, Conn.) for 30 min. The pellet was suspended in sterile distilled water, and the procedure was repeated three or four times. Following a final centrifugation, the cell pellet was suspended in 5 ml of sterile distilled water and sonicated to 90% disruption. Ion chromatography was performed with a Dionex (Sunnyvale, Calif.) chromatograph as previously described by Smith et al. (23, 24). The identity of each peak in the chromatogram was verified by spiking the sample with known standards. The ion concentrations were divided by the length of the cells in each sample to account for an increase in concentration that might result from an increase in cell size.

RESULTS

Synchronized cells in three different sporulation media. Initial experimentation with different sources of carbon revealed that the highest percentage of sporulation was obtained in cells that were synchronized by sequential transfer with dilution, followed by subsequent dilution into xylan sporulation medium supplemented with excess inorganic or organic calcium. Greater than 90% of the synchronized cells elongated a minimum of fourfold in the final batch culture and eventually developed into refractile sporangia (data not shown). However, when the cells were cultured for a sufficient time to permit complete differentiation of the elongated cells into refractile spores, cycles of sporulation and germination were observed.

To overcome this problem of germination, the cells were synchronized in the xylan medium as described above and cultured in sporulation medium containing either xylan with excess Ca carbonate, xylan with excess Ca gluconate, or Ca gluconate alone. At 45 h, the cell density in each of the media tested reached approximately 1.0×10^9 cells per ml and greater than 65% of the cells elongated a minimum of fourfold. Of the elongated cells, more than one-half were further elongated (10- to 20-fold), with evenly spaced multisepta. In addition, 10 to 15% of the cells that were cultured in either of the sporulation media containing xylan showed signs of terminal swelling. When these cultures were shifted to the restrictive temperature (35°C) and incubated for an additional 100 to 155 h, the cells that were highly elongated continued to divide in such a manner that they remained in an elongated state (fourfold the length of vegetative cells or longer) after septation was complete. An increase in cell

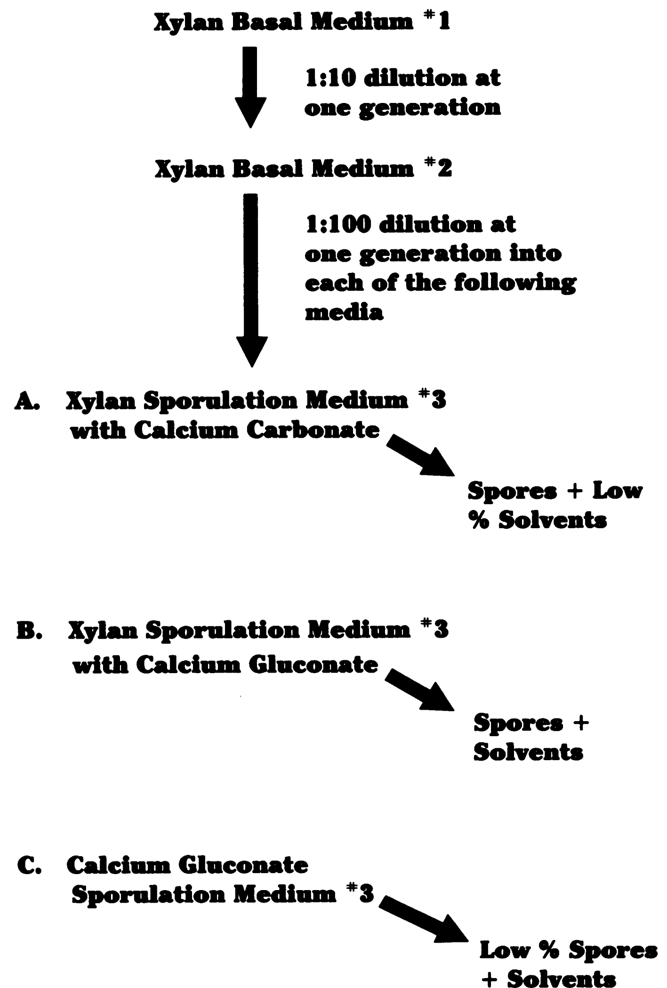

FIG. 1. Culture conditions used to bring about different paths of sporulation and solventogenesis in synchronized, elongated cells of *C. thermosaccharolyticum*.

number (1.0×10^9 to 5.4×10^9 in the xylan-Ca gluconate medium), therefore, was observed and differentiation continued, even at 35°C. Differential counting at the end of the incubation period (205 h) revealed that 89.6% of the total population was converted to refractile, stain-resistant spores in this same medium.

Table 1 summarizes the results of the temperature shift experiments. There was no evidence of germination in any of the spores prepared by this technique, which had been observed in the initial studies with no temperature shift. The refractile sporangia lysed during the storage period at 4°C, releasing free, refractile, stain-resistant spores, but there was no change in the total cell count or the total spore count, even after 6 months of storage.

A solventogenic stage preceding formation of refractile spores. Gas-liquid chromatographic analysis of the metabolic end products of synchronized, elongated cells in the sporulation media revealed a high degree of solventogenesis. The combined concentration of butanol, ethanol, and isopropanol was 0.36% at 2 h, with no detectable acids. These cells continued to produce alcohols, even after the temperature shift. The highest concentration of total solvents (9.63%) was obtained after 95 h of incubation at the restrictive temperature (i.e., 140 h of total incubation time) in the

TABLE 2. Comparison of solvent productivity of *C. thermosaccharolyticum* cells grown under different culture conditions

Organism (medium)	Cellular solvent production rate	
	g of solvent/cell/h	g of solvent/g of cells/h
C. thermosaccharolyticum (xylan-Ca gluconate)	5.90×10^{-12}	7.34×10^{0}
C. thermosaccharolyticum (xylan-$CaCO_3$)	4.62×10^{-14}	4.97×10^{-2}
C. thermosaccharolyticum (Ca gluconate)	1.02×10^{-12}	1.10×10^{0}
C. thermosaccharolyticum NCA 3814[a]	1.07×10^{-13}	1.54×10^{-1}
C. thermosaccharolyticum mutant SD105[a]	8.45×10^{-12}	9.08×10^{0}
Saccharomyces cerevisiae[b]	1.00×10^{-11}	4.00×10^{-1}

[a] Reference 15.
[b] Reference 12.

xylan-Ca gluconate medium. This correlated with a peak in the number of sporangia (83% of the total cell count). Throughout the remainder of the incubation period, as the sporangia became refractile and free spores were released, the corresponding solvent production fell. Interestingly, the butanol-to-ethanol ratio during the peak of solventogenesis was 2:1. However, when differentiation to refractile spores was complete, the ratio was 1:1.5.

The elongated, synchronized cells developed differently, depending on the constituents of the sporulation medium (Fig. 1). For example, cells grown in xylan-$CaCO_3$ medium produced 17.0% free refractile spores with no significant concentration of solvents (0.17%). In medium 2 (xylan-Ca gluconate), cells produced not only a high percentage of refractile spores (89.6%) but also a high concentration of solvents (9.63%). Virtually 100% of the synchronized, elongated, solventogenic cells entered the sporangial stage and continued differentiation through the later stages of sporulation to become spores. In medium 3, containing Ca gluconate as the sole source of carbon, the cells remained elongated, without terminal swelling, and were solventogenic (4.6% total solvents) but did not produce free spores to any significant extent (2.7%).

As indicated by the calculations presented in Table 2, the cells grown in media 2 and 3 produced a significant amount of solvents. However, the productivity of the cells in the xylan-Ca gluconate medium was seven times greater than that of cells in medium that contained only Ca gluconate. In addition, the productivity of cells cultured in the two media that contained Ca gluconate was significantly higher than most reported in other investigations (12, 15, 16).

Calcium incorporation by elongated cells. The data presented in Table 3 were generated from cells grown in medium without supplemented calcium (the basal medium contained 0.001 M $CaCl_2 \cdot 2H_2O$). Short, vegetative cells (one- to twofold normal length) incorporated approximately one-half of the calcium that elongating cells (three- to fourfold normal length) incorporated. The elongated, highly solventogenic cells (≥fourfold normal length) incorporated nearly 10 times as much calcium. When the calcium incorporation of these cells was divided by their length (adjusted for cell length), the elongated, solventogenic cells incorporated more than twice as much calcium as the short, vegetative cells.

TABLE 3. Calcium incorporation by cells of *C. thermosaccharolyticum*

Cell type (length [fold])[a]	Amt of Ca^{2+} incorporated (nmol/10^9 cells)	Amt of Ca^{2+} incorporated (nmol/10^9 cells)/unit of cell length
Short, vegetative (1–2)	9.17	4.59–9.17
Elongating (3–4)	18.20	4.55–9.10
Elongated, solventogenic (≥4)	86.10	17.22–21.53

[a] Cells were grown in basal medium without supplemented excess calcium (basal medium contained 0.001 M $CaCl_2 \cdot 2H_2O$).

When the basal medium was supplemented with excess calcium, the calcium appeared to stabilize the solventogenic cells during the processes of differentiation (data not shown). Lysis of solventogenic cells was never observed in calcium-supplemented cultures, even after 6 months of storage at 4°C. On the other hand, sporulating, solventogenic cells without supplemented calcium deteriorated and lysed within 3 days. A report regarding detailed ion chromatographic analysis of the stabilized, solventogenic cells grown in xylan sporulation medium supplemented with excess calcium is in preparation.

DISCUSSION

It has been observed that spore formation in the genus *Clostridium* is initiated by conversion of highly motile vegetative rods into elongated or swollen, club-shaped clostridial forms that may accumulate granulose, (a polyglucan storage polymer) produce capsules or slime layers, and lose motility (1, 4, 13, 17–19, 22, 28). It has also been noted that the optical density of a culture may continue to increase for some time with no increase in cell number. This increase has been reported to be due to elongation of individual cells without appreciable cell division, increase in cell size (swelling), accumulation of storage granules, or changes in the optical properties of the sporulating cells (10, 11, 13). Furthermore, in many clostridia, spore formation appears to require an exogenous carbon and energy supply throughout the entire sporulation cycle (4, 10, 11, 22, 28).

The probability that vegetative cells will sporulate also appears to be related to the growth rate. Extensive sporulation occurs only in cultures with decreased growth rates (4, 5, 10, 11, 28). In addition, there is evidence that the cells are competent to initiate sporulation only at a specific stage of the DNA replication cycle (3, 5). Mandelstam (20) reported on synchronized cells of *Bacillus subtilis* and emphasized that there was a peak of susceptibility to induction of sporulation.

Our results demonstrated that only cells which were sequentially diluted into fresh medium at each generation, with a final dilution into a xylan sporulation medium containing excess calcium, elongated, developed into clostridial forms, and continued to differentiate into refractile, stain-resistant spores. This correlates well with previous findings (4, 8, 9, 11) and implies that there is a critical time in the division cycle when cells can be induced to sporulate at a high frequency. However, cells which fail to elongate to the critical length (fourfold the length of vegetative cells) also fail to develop into sporangia.

The relationship between solvent formation and sporulation in *Clostridium* spp. has also been recognized and investigated in both batch and chemostat cultures (6, 7, 13, 14, 17–19). During conventional acetone-butanol fermentation, the onset of solvent production is known to be associated with a reduction in growth rate (2, 25), and the link

between the switch to solvent production and the end of exponential growth has been demonstrated (28). In addition, cessation of cell division was frequently followed by the rapid onset of sporulation; greater than 90% of the cells developed forespore septa during the solvent-producing phase (17–19). This correlation was strengthened by selection of *cls* mutants that were unable to form a clostridial stage, produce granulose, form capsules, produce endospores, or switch to solventogenesis, and *spo* mutants that were blocked at a later stage in sporogenesis and did switch to solventogenesis (13, 19). Recently, an asporogenic mutant of *C. thermocellum* was isolated that produced ethanol (up to 1.27 g from 48.3 g of cellulose per liter) as the main product of cellulose degradation after 3 days of culturing (26, 27).

This investigation showed that synchronized, elongated cells of *C. thermosaccharolyticum* were solventogenic in all of the sporulation media tested. However, the highest total concentration of butanol-ethanol-isopropanol (9.63%) was obtained in cells which were synchronized in xylan medium and diluted into xylan-Ca gluconate sporulation medium. Solventogenesis appeared to continue as long as the cells remained elongated and nonrefractile. As the sporangia differentiated, the solvent concentration, particularly that of butanol, decreased.

The data reported here suggest the following. (i) Only cells synchronized by sequential dilution at a critical time (one generation) are induced to elongate, metabolize carbon to solvents, and form refractile, stain-resistant endospores. (ii) One hundred percent of the synchronized, elongated, solventogenic cells developed into refractile spores in the medium containing xylan and Ca gluconate. (iii) The calcium dependence is consistent with previous findings suggesting that procaryotic cells require Ca^{2+} to achieve maximal rates of proteolysis, an early event of sporulation in *B. subtilis* (21).

The solventogenic stage, therefore, appears to be an essential step in sporulation of *C. thermosaccharolyticum*.

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REFERENCES

1. **Awang, G. M., G. A. Jones, and W. M. Ingledew.** 1988. The acetone-butanol-ethanol fermentation. Crit. Rev. Microbiol. **15:** 533–567.
2. **Davies, R., and M. Stephenson.** 1941. Studies on the acetone-butyl alcohol fermentation. I. Nutritional factors involved in the preparation of active suspension of *Clostridium acetobutylicum* (Weizmann). Biochem. J. **35:**1320–1331.
3. **Dunn, G., P. Jeffs, N. H. Mann, D. M. Torgersen, and M. Young.** 1978. The relationship between DNA replication and the induction of sporulation in *Bacillus subtilis*. J. Gen. Microbiol. **108:**189–195.
4. **Ellar, C., and Z. J. Ordal.** 1972. Interference-contrast and phase-contrast microscopy of sporulation in *Clostridium thermosaccharolyticum* grown under strict anaerobiosis. J. Bacteriol. **111:**674–681.
5. **Freese, E.** 1981. Initiation of bacterial sporulation, p. 1–12. *In* H. Levinson, A. L. Sonenshein, and D. J. Tripper (ed.), Sporulation and germination. American Society for Microbiology, Washington, D.C.
6. **Gottschal, J. G., and J. G. Morris.** 1981. The induction of acetone and butanol production in cultures of *Clostridium acetobutylicum* by elevated concentrations of acetate and butyrate. FEMS Microbiol. Lett. **12:**385–389.
7. **Gottschal, J. C., and J. G. Morris.** 1981. Nonproduction of acetone and butanol by *Clostridium acetobutylicum* during glucose-limitation and ammonium-limitation in continuous culture. Biotechnol. Lett. **3:**525–530.
8. **Hoffmann, J. W., E. K. Chang, and E. J. Hsu.** 1978. Interruption in cell division by catabolite dilution producing synchronous growth of *Clostridium thermosaccharolyticum*, p. 312–318. *In* G. Chambliss and J. C. Vary (ed.), Spores VII. American Society for Microbiology, Washington, D.C.
9. **Hsu, E. J.** 1976. Synchronous elongation of *Clostridium thermosaccharolyticum* and its relation to stage I of sporulation, p. 223–242. *In* G. H. Gould and J. Wolf (ed.), Spore research. Academic Press, Inc., New York.
10. **Hsu, E. J., and Z. J. Ordal.** 1969. Sporulation of *Clostridium thermosaccharolyticum* under conditions of restricted growth. J. Bacteriol. **97:**1511–1512.
11. **Hsu, E. J., and Z. J. Ordal.** 1969. Sporulation of *Clostridium thermosaccharolyticum*. Appl. Microbiol. **18:**958–960.
12. **Inloes, D. S., D. P. Taylor, S. N. Cohen, A. S. Michaels, and C. R. Robertson.** 1983. Ethanol production by *Saccharomyces cerevisiae* immobilized in hollow fiber membrane reactions. Appl. Environ. Microbiol. **46:**264–278.
13. **Jones, D. T., N. Van der Westhuizen, S. Long, E. R. Allcock, S. J. Ried, and D. R. Woods.** 1982. Solvent production and morphological changes in *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **43:**1434–1439.
14. **Jones, D. T., and D. R. Woods.** 1986. Acetone-butanol fermentation revisited. Microbiol. Rev. **50:**484–524.
15. **Landuyt, S. L., and E. J. Hsu.** 1985. Solvent fermentation precedes acid fermentation in elongated cells of *Clostridium thermosaccharolyticum*, p. 477–493. *In* G. J. Dring, D. J. Ellar, and G. W. Gould (ed.), Fundamental and applied aspects of bacterial spores. Academic Press, Inc. (London), Ltd., London.
16. **Landuyt, S. L., E. J. Hsu, and M. Lu.** 1983. Transition from acid fermentation to solvent fermentation in a continuous dilution culture of *Clostridium thermosaccharolyticum*. Ann. N.Y. Acad. Sci. **413:**473–478.
17. **Long, S., D. T. Jones, and D. R. Woods.** 1983. Sporulation of *Clostridium acetobutylicum* P262 in a defined medium. Appl. Environ. Microbiol. **45:**1389–1393.
18. **Long, S., D. T. Jones, and D. R. Woods.** 1984. Initiation of solvent production, clostridial stage and endospore formation in *Clostridium acetobutylicum* P262. Appl. Biotechnol. **20:**256–261.
19. **Long, S., D. T. Jones, and D. R. Woods.** 1984. The relationship between sporulation and solvent production in *Clostridium acetobutylicum* P262. Biotechnol. Lett. **6:**529–534.
20. **Mandelstam, J.** 1976. Bacterial sporulation: a problem in biochemistry and genetics of a primitive developmental system. Proc. R. Soc. London Sect. B Biol. Sci. **193:**89–106.
21. **O'Hara, M. B., and J. H. Hageman.** 1990. Energy and calcium ion dependence of proteolysis during sporulation of *Bacillus subtilis* cells. J. Bacteriol. **172:**4161–4170.
22. **Pheil, C. G., and Z. J. Ordal.** 1967. Sporulation of the "thermophilic anaerobes." Appl. Microbiol. **15:**893–898.
23. **Smith, R. E., S. Howell, and D. Yourtee.** 1988. Ion chromatographic determination of sugar phosphates in physiological samples. J. Chromatogr. **439:**83–92.
24. **Smith, R. E., and R. A. MacQuarrie.** 1989. Determination of inositol phosphates and other anions in rat brain. J. Chromatogr. Sci. **27:**491–495.
25. **Spivey, M. J.** 1978. The acetone/butanol/ethanol fermentation. Process Biochem. **13:**2–5.
26. **Tailliez, P., H. Girard, R. Longin, P. Beguin, and J. Millet.** 1989. Cellulose fermentation by an asporogenous mutant and ethanol-tolerant mutant of *Clostridium thermocellum*. Appl. Environ. Microbiol. **55:**203–206.
27. **Tailliez, P., H. Girard, J. Millet, and P. Beguin.** 1989. Enhanced cellulose fermentation by an asporogenous mutant and ethanol-tolerant mutant of *Clostridium thermocellum*. Appl. Environ. Microbiol. **55:**207–211.
28. **Woods, D. R., and D. T. Jones.** 1986. Physiological response of *Bacteroides* and *Clostridium* strains to environmental stress factors. Adv. Microbiol. Physiol. **28:**1–64.