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Extracellular Endo-β-1,4-Glucanase in *Cellvibrio vulgaris*

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Endo-β-1,4-glucanase of the cellulolytic bacterium *Cellvibrio vulgaris* is an actively secreted, truly extracellular enzyme, as supported by growth and secretion studies using filter paper as the sole carbon source.

Little is known of the location of the two major classes of cellulases in bacteria. No satisfactory isolation of the exo-β-1,4-glucanase (EC 3.2.1.21) has been demonstrated and a tight membrane association is thought to exist (1). The endo-β-1,4-glucanase (CMCase) component (EC 3.2.1.4) of cellulase, on the other hand, has been found repeatedly in culture filtrates (2-4, 9). Nevertheless, no conclusive evidence has been presented to support a truly extracellular, actively secreted character for this enzyme. Berg (1) found extracellular enzyme activity to increase parallel to growth, but maintained that this phenomenon is largely accounted for by lysis of cells over the long incubation period. The enzyme in the filtrate has generally been termed "cell-free." This paper demonstrates that when cellulose is the sole carbon source for *Cellvibrio vulgaris*, extracellular endoglucanase is secreted actively in the growth phase.

The strain of *C. vulgaris* used in this study (QM2) was provided by Hillel Levinson, U.S. Army Natick Development Center (Natick, Mass.). The organism was grown in 125-ml Erlenmeyer flasks containing 30 ml of *Cellvibrio* salts (2) and 0.4% untreated Whatman filter paper. Cultures were grown at room temperature (21°C) on a reciprocal shaker. (Although optimum temperature for enzyme production was found to be 30°C, to run large-scale batch cultures a platform shaker at room temperature was employed.) Eight 2.5-liter Fernbach flasks containing 1.0 liter of medium each, as described above, were inoculated with 10 ml of a 6-day culture of *C. vulgaris*. At various times (days 0, 1, 2, 3, 4, 6, 8, and 15), one flask was removed from the shaker and treated as follows.

Extracellular fraction. The extracellular fraction was obtained by centrifuging whole culture at 10,000 × *g* (4°C) for 15 min (IEC refrigerated centrifuge, model B-20). The supernatant fluid was decanted and used as the extracellular fraction. The remainder of the culture (pellet) was used as described in the following procedures for preparation of the "cell-bound" and "intracellular" fractions.

Cell-bound fraction. The cell-bound or periplasmic fraction was prepared by suspending half of the pellet described above in 50 ml of the following preparation: lysozyme (0.05 mg/ml of cell suspension) in 0.025 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.0) containing 5 mM ethylenediaminetetraacetic acid and 0.5 M sucrose. This suspension was gently agitated for 30 min at 30°C. The sample was then centrifuged at 4°C and 10,000 × *g* for 15 min. The well-drained pellet was rapidly dispersed in 80 ml of cold $MgCl_2$ (5×10^{-4} M) solution, agitated gently for another 10 min, and centrifuged as above for 15 min. According to Heppel (7) this "shock fluid" (supernatant) contains the hydrolytic enzymes of the periplasmic space.

Intracellular fraction. The intracellular fraction was obtained by sonic disruption of half of the pellet from the extracellular fraction (suspended in 40 ml of 0.067 M KH_2PO_4-Na_2HPO_4 buffer, pH 7.0) at 7 kHz for 15 min. The sample was then centrifuged at 10,000 × *g* for 20 min, and the supernatant fluid was retained as the intracellular fraction. Microscopic examination showed that over 95% of the cells were disrupted. Samples were stored at −70°C and were stable for over 2 months.

It is recognized that the two methods above are not mutually exclusive, but do represent enrichment of one fraction or the other.

Protein determination. Protein determination was by the method of Lowry et al. (8). Cell protein was obtained by sonically disrupting *C. vulgaris* cells (centrifuged at 10,000 × *g*, washed, and resuspended in phosphate buffer, pH 7.0) at 7 kHz for 10 min. A standard curve was prepared using bovine serum albumin.

DNA determinations. DNA determination was carried out by Burton's diphenylamine method (5) on the soluble fraction after sonic disruption of cells. Purified calf thymus DNA

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was employed as the standard.

Enzyme assay. The substrate used throughout was Hercules carboxymethylcellulose, (degree of substitution, 0.4; Hercules Powder Co., Wilmington, Del.) as a 1% solution in 0.067 M phosphate buffer (pH 7.0). Sodium azide (0.01%) was added as a preservative. The assay was performed as described previously (10). Activity (international units) was expressed as 1 μmol of reducing sugar (as glucose) produced per min under standard conditions.

Experiments performed to determine the effect of chloramphenicol as an inhibitor of de novo protein synthesis employed a "secretion medium" consisting of 0.067 M phosphate buffer (pH 7.0), 1 mM $MgSO_4 \cdot 7H_2O$, and 0.4% Whatman no. 1 filter paper. Twenty-nine milliliters was distributed to 50-ml Fernbach flasks and then autoclaved. *C. vulgaris* cells, grown on filter paper medium (as previously described) for 6 days, centrifuged, and resuspended in buffer, were employed as the inoculum. Cell suspension (1 ml) was added aseptically to each of the flasks, and the cultures were kept at 30°C in a reciprocal shaker-water bath. Chloramphenicol (Sigma Chemical Co., St. Louis, Mo.) was prepared fresh each day as a stock solution of 1 mg/ml, filter sterilized, and then added aseptically to the appropriate flask to a final concentration of 10 μg/ml. Samples were removed as required for standard determination of CMCase activity; 1-day intervals were employed, in keeping with the prolonged growth curve that the organism exhibits on insoluble substrate.

Growth of *C. vulgaris* on filter paper displayed a lag phase of approximately 24 h, then a logarithmic phase terminating at day 2, followed by gradual entry (days 3 to 6) into stationary phase, which persisted through day 15, at which point the experiments were terminated. Extracellular CMCase activity increased from day 1 over the entire growth curve, beginning with the onset of logarithmic phase. Activity continued to increase through day 15, although cell protein was constant from day 6 through 15 (Fig. 1).

Figure 2 illustrates the location of CMCase

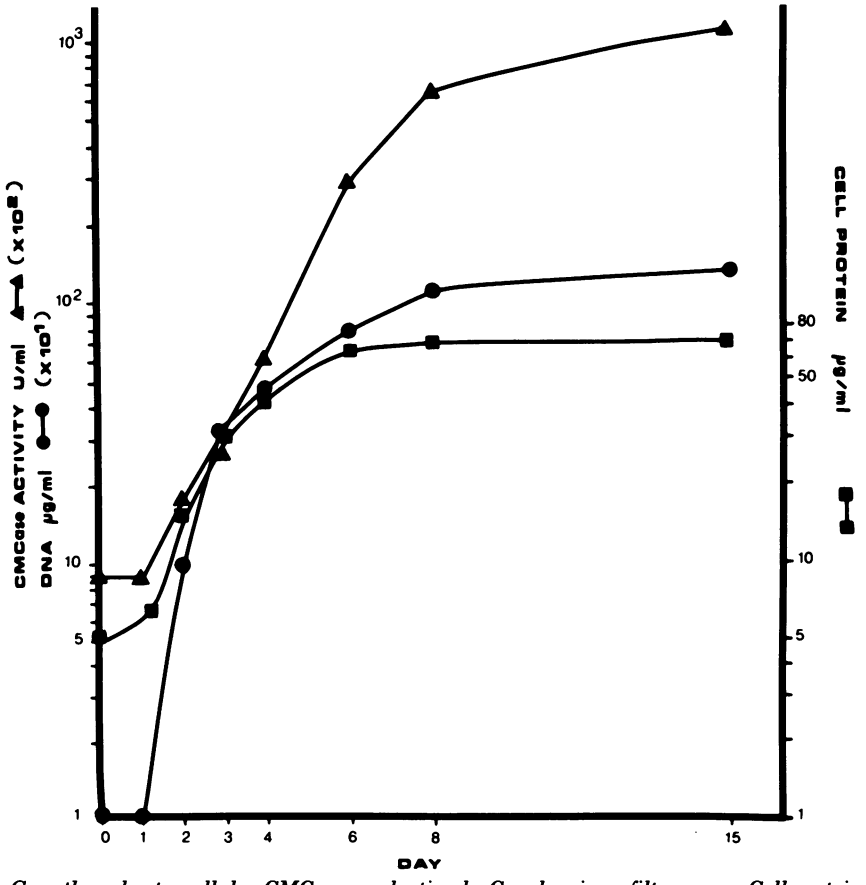

FIG. 1. *Growth and extracellular CMCase production by C. vulgaris on filter paper. Cell protein and DNA are used as growth parameters. Incubated at room temperature (21°C) on a reciprocal shaker. Each point represents the average of three determinations.*

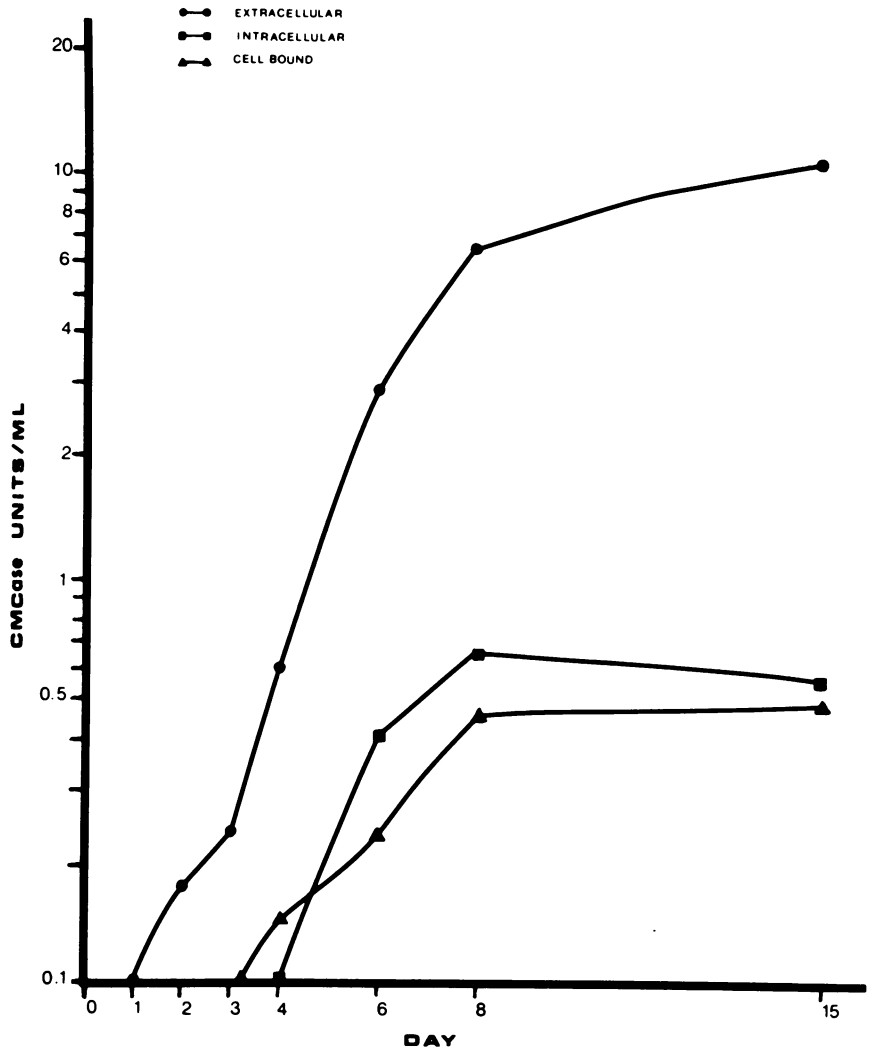

FIG. 2. *Location of CMCase activity from C. vulgaris grown on filter paper. CMCase activity indicated per milliliter of total culture in all cases.*

activity in extracellular, intracellular, and cell-bound fractions. Extracellular activity was consistently at least eight times greater than that associated with intracellular or cell-bound fractions. No detectable activity was observed before day 4 in either cell-bound or intracellular fractions.

The chloramphenicol experiment was modeled after that performed by Stinson and Merrick (11) with *Pseudomonas lemoignei,* in which they hypothesized that if the organism accumulates poly-β-hydroxybutyrate depolymerase during the growth phase, then the secretion phase observed should be insensitive to chloramphenicol. Their results showed secretion to be inhibited upon addition of chloramphenicol at all times tested. This indicated de novo protein synthesis to be necessary for secretion to proceed, assuming the antibiotic itself did not affect lysis. The above secretion medium was modified to contain 0.4% Whatman filter paper as the carbon source, and the time scale of the experiment was expanded to correlate with the normal growth curve of the organism on filter paper. Incubation of washed cells in the above-mentioned secretion medium resulted in contined release of CMCase. Periodic addition of chloramphenicol to the culture effectively inhibited either synthesis or release, or both, at all time intervals examined (Fig. 3).

Cellulases have been found extracellularly in a variety of strains of *Bacillus* (9), whereas organisms such as *Cytophaga* possess only cytoplasmic, periplasmic, or membrane-bound cel-

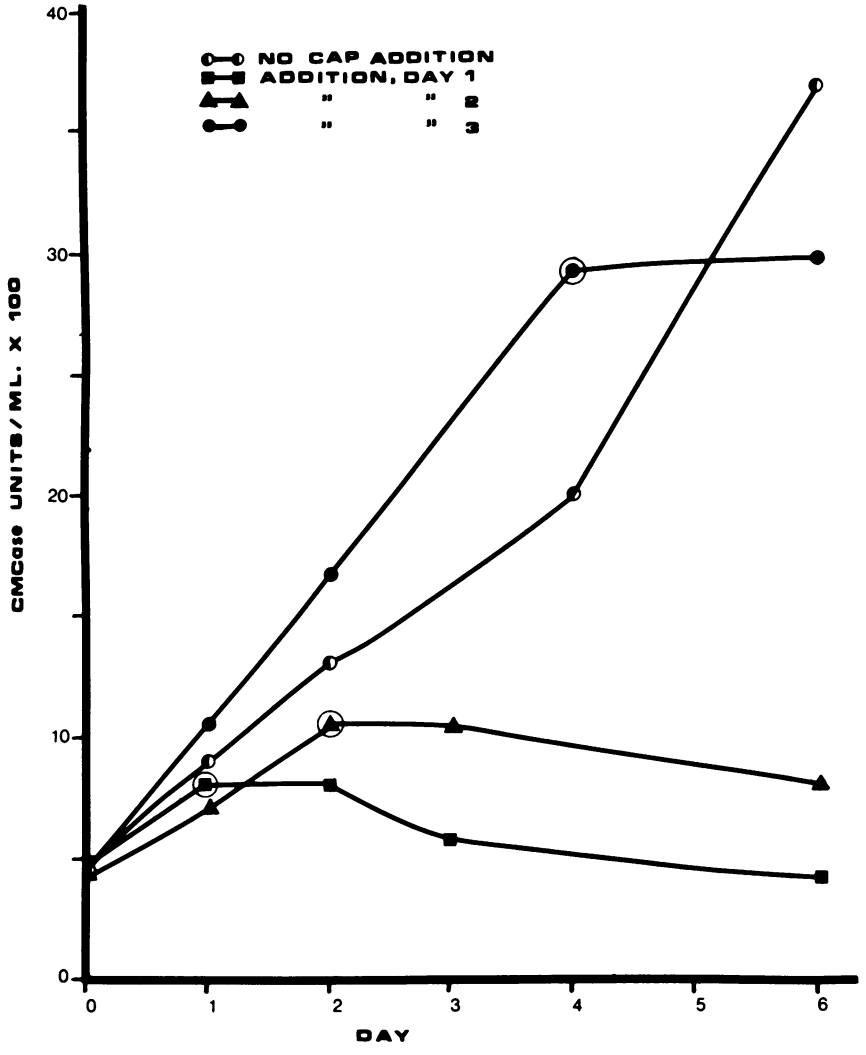

FIG. 3. *Effect of chloramphenicol on synthesis and/or secretion of CMCase with filter paper as substrate. Cells pregrown on filter paper were inoculated into sterile secretion medium, which included 0.4% (wt/vol) filter paper. On days designated, chloramphenicol (CAP; final concentration, 10 μg/ml) was added aseptically. Flasks were incubated at 30°C in a reciprocal shaker-water bath.*

lulases (6). Between these extremes lie the gram-negative, cellulolytic bacteria (*Pseudomonas, Cellulomonas,* and *Cellvibrio*) (1, 2, 12, 13), in which cellulases may appear either predominantly cell associated or cell free, depending upon the organism and its stage of growth. Yamane et al. (12) and Yoshikawa et al. (13), using *Pseudomonas fluorescens* subsp. *cellulosa,* found two cell-free components which they termed extracellular. Whereas filtrate CMCase activity was evident in Berg's studies with *C. fulvus,* this phenomenon was attributed largely to lysis-associated, nonspecific release rather than actual secretion (2).

As emphasized by Priest (9), extracellular or exoenzymes may be extracellular at all phases of growth or may be bound in young cells and released as exoenzymes in the stationary phase. The results presented in this paper suggest that at least some of the extracellular endoglucanases of *C. vulgaris* are of the former type, i.e., are secreted throughout the growth cycle. Extracellular CMCase activity in this organism is evident throughout the growth curve, and its active increase during the log phase further supports the mode of extracellularity described above. Cell fractions examined for enzyme activity reveal localization of endoglucanases predominantly in

the extracellular fraction at all phases of culture growth. Were internal enzyme pools responsible for elevated CMCase levels in the stationary phase, such pools would inflate intracellular fraction values.

The technique of observing washed cells resuspended in fresh medium for the purpose of assessing an enzyme's extracellular secretion has been described several times in the literature (3, 11). In experiments studying the secretion of protease in *Bacillus amyloliquefaciens*, Both et al. (3) ruled out the presence of preformed enzyme accounting for release of protease in this system by using chloramphenicol, which effected a rapid inhibition. Stinson and Merrick (11) likewise found poly-β-hydroxybutyrate depolymerase synthesis and release to be closely coupled in *P. lemoignei* by using the same method. The endoglucanase of *C. vulgaris*, from our present observations, appears to respond to chloramphenicol treatment in an identical manner.

It is concluded from these experiments that *C. vulgaris* elaborates extracellular endoglucanase whose release parallels the growth curve of the organism. De novo protein synthesis and release of this cellulase are coupled, as demonstrated by treatment with chloramphenicol. The data presented support the findings of Yamane et al. (12) and Yoshikawa et al. (13) for *P. fluorescens*, but contrast with the conclusions of Berg et al. for *C. fulvus* (2). The inability of *C. fulvus* to grow on soluble cellulose derivatives such as carboxymethylcellulose, which *C. vulgaris* and *P. fluorescens* can use as a sole carbon source, may offer some explanation of this phenomenon.

LITERATURE CITED

1. **Berg, B.** 1975. Cellulase location in *Cellvibrio fulvus*. Can. J. Microbiol. **21**:51-57.
2. **Berg, B., B. von Hofsten, and G. Pettersson.** 1972. Growth and cellulase formation by *Cellvibrio fulvus*. J. Appl. Bacteriol. **35**:201-214.
3. **Both, G. W., J. L. McInnes, J. E. Hanlon, B. K. May, and W. H. Elliot.** 1972. Evidence for an accumulation of messenger RNA specific for extracellular protease and its relevance to the mechanism of enzyme secretion in bacteria. J. Mol. Biol. **67**:199-207.
4. **Breuil, C., and D. J. Kushner.** 1976. Cellulase induction and the use of cellulose as a preferred growth substrate by *Cellvibrio gilvus*. Can. J. Microbiol. **22**:1766-1781.
5. **Burton, K.** 1956. A study of the conditions and mechanisms of diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315-323.
6. **Chang, W. T. H., and D. W. Thayer.** 1977. The cellulase system of a *Cytophaga* species. Can. J. Microbiol. **23**:1285-1292.
7. **Heppel, L. A.** 1967. Selective release of enzymes from bacteria. Science **156**:1451.
8. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
9. **Priest, F. G.** 1977. Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriol. Rev. **41**:711-753.
10. **Rosenberg, F. A., and L. V. Oberkotter.** 1977. Methods for determining microbial biodegradation in cellulose, p. 243-259. *In* A. H. Walters (ed.), Biodeterioration investigation techniques. Applied Science Publishers, Ltd., Barking-on-Essex.
11. **Stinson, M. W., and J. M. Merrick.** 1974. Extracellular enzyme secretion by *Pseudomonas lemoignei*. J. Bacteriol. **119**:152-161.
12. **Yamane, K., H. Suzuki, M. Hirotani, H. Ozawa, and K. Nisizawa.** 1970. Effect of nature and supply of carbon sources on cellulase formation in *Pseudomonas fluorescens* var. *cellulosa*. J. Biochem. **67**:9-16.
13. **Yoshikawa, T., H. Suzuki, and K. Nisizawa.** 1974. Biogenesis of multiple cellulase components of *Pseudomonas fluorescens* var. *cellulose*. I. Effects of culture conditions on the multiplicity of cellulase. J. Biochem. **75**:531-540.