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Extracellular Enzyme Secretion by *Pseudomonas lemoignei*

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The ability of succinate to repress the secretion of *Pseudomonas lemoignei* poly-β-hydroxybutyrate depolymerase was a function of pH. Repression only occurred when the pH of the medium was 7.0 or less. At a higher pH, lack of sensitivity to succinate concentration may have been due to a limited ability to transport succinate. Actively secreting cultures (at pH 7.4) continued to secrete enzyme for approximately 30 min after the pH was rapidly decreased to pH 6.8, even though sufficient succinate was present to repress enzyme synthesis. Similarly, after the addition of rifampin to secreting cultures, there was a 30-min delay before secretion was inhibited. Evidence is presented which suggests that continued secretion may be the result of depolymerase messenger ribonucleic acid accumulation within the cells. Studies with chloramphenicol indicated that de novo protein synthesis is necessary for the secretion of poly-β-hydroxybutyrate depolymerase and that exoenzyme is not released from a preformed pool. Studies with various inhibitors of protein synthesis indicated that synthesis of exoenzyme is 5 to 10 times more susceptible to inhibition than is the synthesis of cell-associated proteins.

The secretion of extracellular enzymes by bacteria occurs in the absence of cell lysis and involves a highly selective permeation process. A model has been proposed (17) which suggests that synthesis occurs at specific translational sites on the cytoplasmic membrane and is coupled with the simultaneous extrusion of the growing peptide chain into the external environment. Recent studies on enzyme secretion by *Bacillus amyloliquefaciens* have shown that under certain conditions, secreting cells accumulate large pools of messenger ribonucleic acid (mRNA) which are capable of supporting enzyme synthesis and secretion for relatively long periods of time in the presence of rifampin or actinomycin D (2, 8, 10). It was speculated that the overproduction of mRNA is related to the mechanism of extracellular enzyme synthesis which ensures that sufficient mRNA reaches translation sites despite its rapid breakdown during transport from gene to membrane.

Many exoenzymes are synthesized and secreted toward the end of exponential growth; however, the regulatory mechanisms which control the production of these enzymes are little understood. Studies by Tanaka and Iuchi (30) on the synthesis of an extracellular proteinase by *Vibrio parahaemolyticus* suggested that the repression is similar to catabolite repression, but they noted certain differences since the repression was not reversed by cyclic adenosine 3',5'-monophosphate (c-AMP).

Enzyme secretion appears to be more common in gram-positive than in gram-negative bacteria, a property which may be a reflection of the complex outer-membrane structures of gram-negative organisms. It does occur in certain pseudomonads and vibrios (22), but very few detailed studies on enzyme secretion by these organisms are available. To investigate the nature of extracellular enzyme synthesis and secretion by gram-negative bacteria, we initiated studies with *Pseudomonas lemoignei*, an organism which constitutively secretes a poly-β-hydroxybutyric acid (PHB) depolymerase. The properties of this organism and the secreted enzyme have been previously described (5, 6, 15).

MATERIALS AND METHODS

Antibiotics. The antibiotics used in this study were chloramphenicol (Sigma Chemical Co., St. Louis, Mo.), puromycin (Nutritional Biochemical Corp., Cleveland, Ohio), rifampin (Calbiochem, San Diego, Calif.), and streptovaricin (generously donated by the Upjohn Co., Kalamazoo, Mich.).

PHB granules. The PHB granules used for the enzymatic assays were obtained as purified inclusion granules from *Bacillus megaterium* KM cells grown in a medium containing 0.3% glucose and 0.05 M sodium acetate as described by Macrae and Wilkinson (16).

During late exponential growth, the bacteria were harvested by centrifugation and the PHB inclusion granules were isolated and purified by the procedures of Delafield et al. (5). Stable suspensions of the purified polymer were prepared in distilled water (5 mg/ml) by ultrasonication (Biosonic III, Bronwill Instruments).

Organism and cultural conditions. *P. lemoignei* (ATCC 17989), obtained from M. Doudoroff, was grown in the following minimal medium (modified from Delafield et al. [4]): sodium succinate (15 mM), KH_2PO_4-Na_2HPO_4, pH 6.8 (33 mM), NH_4Cl (18 mM), $MgSO_4 \cdot 7H_2O$ (2 mM), $FeCl_3 \cdot 6H_2O$ (0.037 mM) and $CaCl_2$ (.045 mM). Cultures (400 ml of medium in 2-liter flasks) were incubated on a New Brunswick gyrotary shaker at 30 C until late exponential growth, generally 16 to 18 hours. Growth was followed by measuring turbidity with a Klett-Summerson photoelectric colorimeter (no. 66 filter). Cultures with optical densities between 100 and 120 Klett units were harvested by centrifugation, and the cell pellets were washed twice with 33 mM KH_2PO_4-Na_2HPO_4 buffer (pH 6.8) and then resuspended in the following secretion medium: KH_2PO_4-Na_2HPO_4 (33 mM), pH 6.8, NH_4Cl (18 mM), $MgSO_4 \cdot 7H_2O$ (1 mM), and sodium succinate (12 mM). Washed cells were resuspended in the secretion medium to 500 Klett units (5×10^9 cells/ml; 0.8 mg (dry weight)/ml), and 50-ml samples of the resulting suspension were shaken in 250-ml Erlenmeyer flasks at 30 C.

Enzyme assays. At various intervals during the incubation, 2-ml samples of the cell suspension were withdrawn and centrifuged for 2 to 3 min at 39,000 $\times$ *g*, and the clear supernatant fluids were assayed for PHB depolymerase activity by the turbidimetric method of Delafield et al. (5). Determinations were made by following the OD 660 decrease at 25 C with a Zeiss PM QII spectrophotometer. The reaction mixture contained 250 μg of purified polymer [2.9 μeq of D(−)-β-hydroxybutyric acid (BHB)], tris(hydroxymethyl)aminomethane-H_2SO_4 buffer (50 mM) at pH 8.0, and $CaCl_2$ (1 mM), in a final volume of 1.5 ml. One unit of depolymerase activity is defined as that quantity of enzyme which causes a decrease in turbidity of 0.001 OD unit per minute.

BHB dehydrogenase and BHB dimer hydrolase activities were determined by the procedures described by Shuster and Doudoroff (28) and Delafield et al. (4), respectively. Samples of cell suspensions were centrifuged, washed with 0.05 M Tris hydrochloride buffer at pH 8.0, and resuspended in 2 ml of the same buffer. The cell suspension was disrupted ultrasonically and centrifuged for 15 min at 39,000 $\times$ *g*, and the supernatant fluids were removed for assay. One unit of BHB dehydrogenase is defined as the amount of enzyme that catalyzes the reduction of 1 μmol of nicotinamide adenine dinucleotide (NAD) in 1 min at 25 C. One unit of hydrolase activity is defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of dimer to 2 μmol of monomer per min at 25 C.

Protein synthesis. Total protein synthesis was determined by measuring the incorporation of L-[4,5-^{3}H]leucine (30 Ci/mmol; International Chemical and Nuclear Corp.) into trichloroacetic acid-insoluble material. Washed cells were incubated in secretion medium containing 0.2 μCi of [^{3}H]leucine and 1 μg of unlabeled leucine per ml. At appropriate times, 1-ml samples of a secreting cell suspension were added to an equal volume of 10% trichloroacetic acid and placed in a boiling water bath for 15 min. The samples were cooled to room temperature and centrifuged in a swinging bucket at 1,000 $\times$ *g* for 20 min. The resultant pellets were washed twice with 15 ml of distilled water, dissolved in 0.5 ml of Soluene-100 (Packard Instrument Co.), and quantitatively transferred to scintillation vials. Radioactivity was determined by liquid scintillation counting in a Nuclear Chicago Mark II spectrometer. The liquid scintillation solvent contained 4.9 g of 2,5-diphenyloxazole and 0.1 g 1,4-bis-2-(4-methyl-5-phenyloxazoly')-benzene per liter of toluene.

RNA synthesis. RNA synthesis was determined by measuring [2-^{14}C]uracil (62 mCi/mmol; Amersham-Searle) incorporation into total cellular RNA. Washed cells were incubated in secretion medium containing 0.8 μCi of [^{14}C]uracil and 2 μg of unlabeled uracil per ml. At various time intervals, 1-ml samples were added to tubes maintained at 0 C and containing 1% Casamino Acids and 1 mg of unlabeled uracil per ml in 2 ml of 5% trichloroacetic acid (17). The resulting cell suspensions were held on ice for 30 min and then centrifuged at 1,000 $\times$ *g* for 20 min. The pellets were washed with 5 ml of cold 5% trichloroacetic acid-amino acid mixture twice and then with 5 ml of 1% acetic acid. The preparations were filtered through membrane filters (Millipore Corp.), which were subsequently dried at 80 C and transferred to scintillation vials for radioactive counting.

Other assays. After removal of cells by centrifugation, succinate was determined polarographically (3) in a sealed vessel (29) with a succinoxidase preparation obtained by the procedure described by Umbreit et al. (31). In some experiments, the disappearance of radioactive succinate from the incubation medium was followed by counting the supernatant fluid in a scintillation counter after removal of the bacterial cells.

Protein was measured by the method of Lowry et al. (14), with crystalline bovine serum albumin as standard.

Cell dry weight was determined with distilled water-washed cells dried to constant weight.

RESULTS

P. lemoignei cells, growing in a succinate-mineral salts medium, constitutively secreted extracellular PHB depolymerase, with maximal production occurring toward the end of exponential growth (Fig. 1). The appearance of exoenzyme at the end of exponential growth has been frequently observed in other bacterial systems (12, 22); however, the mechanism of repression during the early stages of growth is not well understood. The correlation of maximal depolymerase production with the depletion of

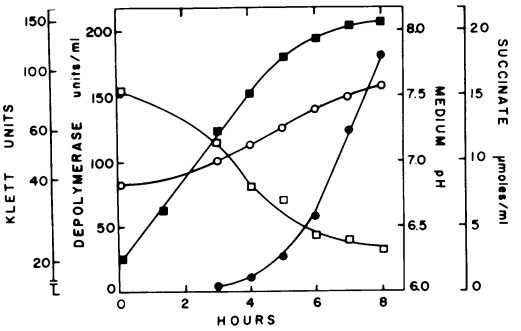

FIG. 1. *Secretion of PHB depolymerase by growing cultures of P. lemoignei. Extracellular PHB depolymerase, ●; pH of medium, ○; Klett, ■; succinate concentration, □.*

succinate from the medium suggests that exoenzyme synthesis and secretion may be regulated by classical catabolite repression. The pH of the culture medium increased from 6.8 to 7.6 during growth and was approximately 7.2 at the onset of enzyme secretion (Fig. 1). It will be shown later that the pH of the medium profoundly affected enzyme secretion.

Two intracellular enzymes have been described which are related to PHB metabolism (4): a BHB dehydrogenase and a hydrolase that decomposes BHB dimer, the principal end product of depolymerase digestion of PHB. To determine whether any regulatory relationship exists between the enzymes concerned with PHB metabolism, the time course of synthesis of the intracellular enzymes was also examined during the growth of *P. lemoignei*. In contrast to the production of extracellular PHB depolymerase during late growth, the intracellular enzymes (BHB dehydrogenase and BHB dimer hydrolase) appeared throughout the entire growth period. The differential rates of enzyme synthesis (Fig. 2) contrast the late appearance of PHB depolymerase with the constant rates of synthesis of the intracellular enzymes throughout the growth period. These studies suggest that the syntheses of the two intracellular enzymes are regulated independently of depolymerase synthesis.

To facilitate further study of the secretion process, we used washed cell suspensions at high cell density under conditions where substantial rates of enzyme secretion are obtained over relatively short periods of time. Maximal secretion required succinate, NH_4^+, and Mg^{2+} in the medium. Omission of any one of these components resulted in insignificant secretion. Of a number of divalent cations tested, only Zn^{2+} and Mn^{2+} were able to replace Mg^{2+} in the secretion medium. At 1 mM, Zn^{2+} was 91% and Mn^{2+} 69% as effective as Mg^{2+}. Ca^{2+}, Co^{2+}, Cu^{2+}, and Sr^{2+} were completely ineffective. The inability of Ca^{2+} to replace Mg^{2+} in the secretion medium suggests that the latter ion is involved in the secretion mechanism itself rather than as a requirement for structural integrity of the enzyme; Ca^{2+} can replace Mg^{2+} in maintaining the stability of the already secreted enzyme (5).

Repression of exoenzyme secretion by succinate. In exponentially growing cells, the synthesis and secretion of exoenzyme was markedly repressed (Fig. 1). This frequently observed property of exoenzyme production may be attributed to severe catabolite repression during the early stages of growth when the concentration of the carbon source is relatively high. After its depletion toward the end of exponential growth, the repression was relieved and exoenzyme synthesis and secretion were initiated. Similar results were obtained with washed cells, resuspended at high cell density in the secretion medium (Fig. 3). At low initial succinate concentration (2 mM), enzyme secretion began immediately and no repression was observed. However, when the succinate concentration was increased (6 and 12 mM), there was a lag period before onset of secretion, which was dependent on the succinate concentration.

If exoenzyme synthesis and secretion is controlled by catabolite repression, as suggested by these data, then it should be possible to interrupt the production of depolymerase by the addition of succinate to suspensions of actively secreting cells. Experiments to demonstrate this, however, were unsuccessful. An additional increment of succinate to a culture actively secreting the depolymerase did not result in any

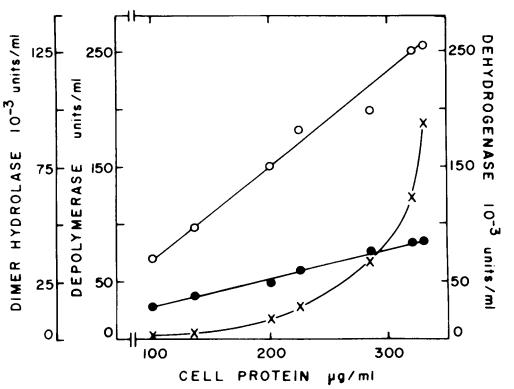

FIG. 2. *Differential rate of enzyme synthesis by growing culture. Extracellular PHB depolymerase, ×; intracellular BHB dimer hydrolase, ●; intracellular BHB dehydrogenase, ○.*

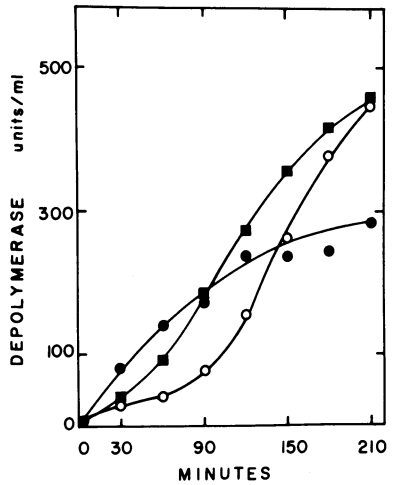

FIG. 3. *Effect of succinate concentration on enzyme secretion. Initial succinate concentration was 2 (●), 6 (■), and 12 mM (○).*

interruption of enzyme production (Fig. 4). Similar results were also obtained with higher increments of succinate (e.g., addition of 24 μmol of succinate per ml at 120 min).

The failure of additional increments of succinate to prevent the production of exoenzyme after the secretion phase had been initiated could have been due to the release of preformed enzyme; that is, PHB depolymerase synthesized and accumulated by growing cells is then released after growth ceases as a result of substrate depletion. Under these conditions, enzyme synthesis and secretion would be uncoupled. An accumulated enzyme pool could be held between the cell wall and cytoplasmic membrane (the periplasmic space), either bound directly to some surface component or unable to diffuse through the complex outer surface layers characteristic of gram-negative bacteria (18). The release of this accumulated enzyme may be facilitated by alterations in the surface of the cells, brought about by depletion of the available carbon source (succinate). Accumulation of a cell-bound enzyme has been shown to occur in the case of penicillinase secretion by *B. licheniformis* (7, 25-27). The release of this enzyme pool occurs in the presence of quantities of chloramphenicol sufficient to completely inhibit protein synthesis. Unfortunately, no reliable method is available to quantitatively measure cell-bound PHB depolymerase since crude extracts of *P. lemoignei* contain an inhibitor of the enzyme (5). However, if cells do accumulate depolymerase during the growth phase, then it might be expected that the secretion phase would be insensitive to chloramphenicol, assuming that the antibiotic does not affect the release mechanism and that it specifically inhibits protein synthesis. The effect of chloramphenicol addition on secretion is seen in Fig. 5. Enzyme secretion was prevented in the presence of chloramphenicol regardless of time of addition, suggesting that de

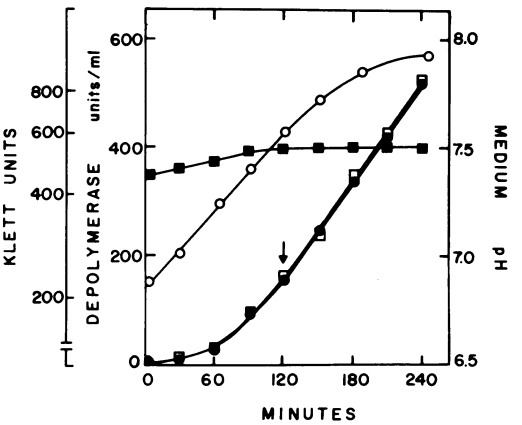

FIG. 4. *Effect of succinate addition on depolymerase production by actively secreting cells. Washed cells were resuspended in secretion medium containing 12 μmol of succinate per ml. At 120 min, an additional 12 μmol of succinate per ml was added (●). Secretion obtained in the absence of any additional succinate, □; Klett, ■; pH, ○.*

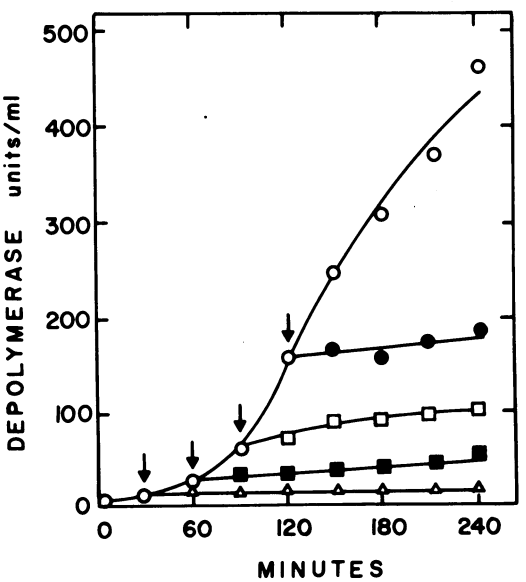

FIG. 5. *Effect of chloramphenicol on depolymerase secretion by washed cell suspensions. Chloramphenicol was added at various time intervals to give a final concentration 10 μg/ml. Chloramphenicol additions: none (○); at 30 min (△); at 60 min (■); at 90 min (□); at 120 min (●).*

novo protein synthesis is required for continued enzyme secretion. It therefore appeared unlikely that the failure of succinate to repress enzyme secretion once it had been initiated was due to the release of a preformed enzyme pool. Thus, as has been found in other microbial systems (9, 17, 26), enzyme secretion and synthesis appear to be tightly coupled.

Effect of pH on enzyme secretion. *P. lemoignei* is restricted to growth on relatively few single carbon sources, namely organic acids such as acetate or succinate. Since the metabolism of salts of these organic acids results in a considerable increase in pH, it is possible that such a marked environmental change may in some way be related to the inability of succinate to repress exoenzyme synthesis during the active secretion phase. In the experiment described in Fig. 4, additional succinate was added at 120 min, at which time the pH had reached 7.6. Growth experiments have established that *P. lemoignei* grows poorly, if at all, at pH 7.2 and above. If one assumes that catabolite repression (or a closely related control mechanism) plays an important regulatory role in exoenzyme synthesis, then it would be expected that any environmental condition which affects the concentration of intermediary metabolites would also have profound effects on the synthesis of PHB depolymerase. The inability of *P. lemoignei* to grow at the higher pH may be due to a succinate transport mechanism that is very sensitive to changes in pH. At the higher pH, succinate may not be transported at a sufficiently rapid rate to permit the synthesis of catabolic pools large enough to allow for cellular growth. Under these conditions, repressing effectors would also be decreased, and this would be reflected in increased exoenzyme synthesis. In support of this possibility, we found that succinate uptake was indeed dependent on pH and decreased with increasing pH (see, for example, Fig. 7B). A transport mechanism directly or indirectly affected by pH would therefore explain the inability of further succinate to prevent exoenzyme synthesis, since the additional increment would not affect an already saturated permeation system proceeding at a suboptimal rate. In experiments where secretion was examined when the pH of the medium was maintained at 7.4 (Fig. 6B), exoenzyme production was immediate and independent of the external succinate concentration, quite unlike the results obtained earlier at pH 6.8 (Fig. 3) or those presented in Fig. 6A. In the latter case, the pH of the medium was maintained constant at 6.8 and succinate concentration was varied. At this pH, enzyme secretion is a function of the succinate concentration. At low succinate concentration (2 mM), the cells secrete enzyme without a detectable lag. Increasing the succinate concentration, however, progressively increases the lag before onset of secretion. Similar studies at pH 7.0 demonstrated that the cells were still repressible by succinate. At higher pH's, however, succinate was no longer able to repress enzyme secretion.

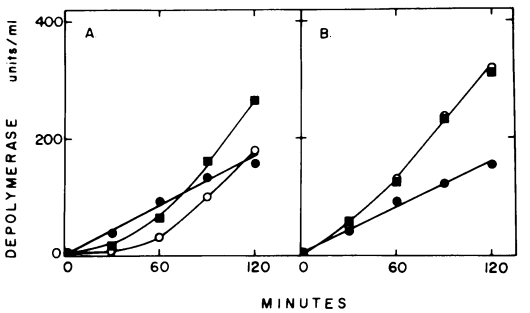

FIG. 6. *Effect of succinate concentration on enzyme secretion at pH 6.8 (A) and 7.4 (B). A constant pH was maintained by continuous addition of HCl. Succinate concentrations: 2 mM (●); 6 mM (■); and 12 mM (○).*

Actively secreting cells at pH 7.4, unaffected by external succinate concentration because of an inefficient transport mechanism, should exhibit prompt repression of exoenzyme synthesis if the pH is reduced to a level where succinate transport becomes sufficiently rapid to increase the level of repressing effectors. The results of such an experiment are seen in Fig. 7. Three washed cell suspensions in secretion medium, containing identical components, were prepared. Secretion was initiated at pH 7.4 in two flasks and at 6.8 in the other. Secretion at pH 7.4 began immediately even at a succinate concentration sufficiently high (10 mM) to repress enzyme synthesis at 6.8 for approximately 90 min (Fig. 7A). The rate of succinate disappearance from the secretion medium (Fig. 7B) was also followed and was considerably more rapid at pH 6.8 than at 7.4. After 120 min of incubation, one of the cultures at pH 7.4 was quickly readjusted to pH 6.8. Succinate uptake in the readjusted medium immediately increased and can be correlated with repression of enzyme secretion, which persisted until it was relieved by the utilization of succinate. It is interesting to note, however, that this repression was not immediate but occurred only after a 30-min delay. A trivial explanation for the failure to cause immediate repression as expected may be that the cells need an appropri-

ate interval to adjust to the altered conditions before the cell can build up repressible levels of catabolic substances. This does not appear to be the case, however, since the bacteria begin to take up succinate at a faster rate immediately after pH adjustment. Similarly the metabolic conversion of succinate into trichloroacetic acid-insoluble substances also increased immediately after pH adjustment (Fig. 7C). It therefore seemed unlikely that 30 min was required to establish the repression. Furthermore, the tight coupling between synthesis and secretion argues against the possibility that the secretion was due to preformed enzyme. It might also be pointed out that derepressed cells at pH 6.8 secrete at about the same rate as derepressed cells at pH 7.4, further suggesting that the effect of pH is at the level of succinate uptake rather than on synthesis and extrusion of exoenzyme.

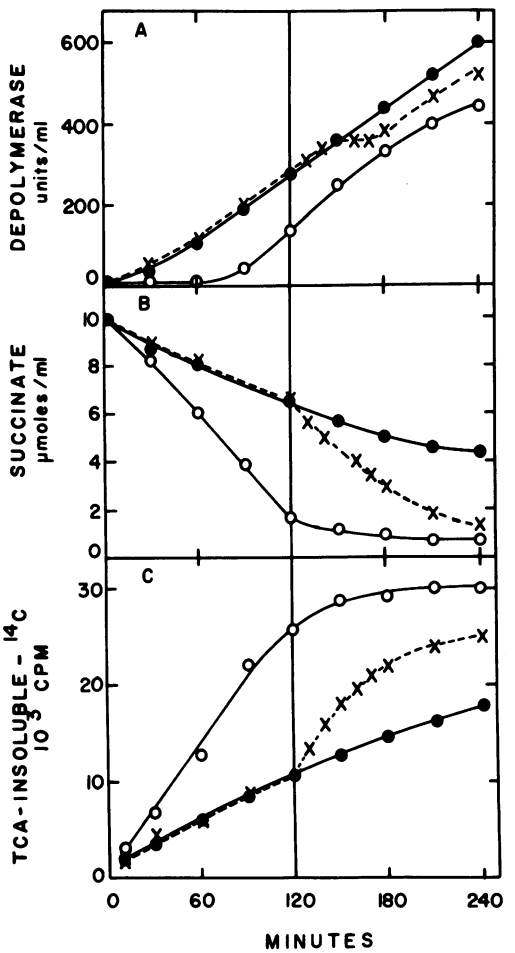

FIG. 7. *Effect of pH on succinate repression of enzyme secretion. Washed cells were resuspended in secretion media at the indicated pH. A constant pH was maintained by the continuous addition of HCl. At 120 min, the pH was rapidly decreased to pH 6.8 in one culture (×) and maintained at this pH throughout the remaining time interval of the experiment. Symbols: ●, pH maintained at 7.4; ○, pH maintained at 6.8. At various time intervals, suitable portions were removed, and (A) assayed for depolymerase activity, (B) succinate disappearance from the medium, and (C) conversion of succinate to trichloroacetic acid-insoluble material. Assays were performed as described in the text.*

Effect of rifampin on enzyme secretion. Continued synthesis of exoenzyme after the adjustment of pH from nonrepressive to repressive conditions was reminiscent of studies carried out by Both et al. (2), Glenn et al. (8), and Gould et al. (10). These workers found that late log-phase cells of *B. amyloliquefaciens* are capable of secreting extracellular proteins for over 60 min in the presence of the RNA synthesis inhibitors rifampin and actinomycin D. Evidence was provided which suggested that synthesis and secretion of exoenzyme under these conditions were due to the presence of a large pool of preformed mRNA whose level was maintained by an excessive rate of transcription rather than due to stable mRNA. They suggest that this hyperproduction of mRNA is related to the mechanism of enzyme secretion and that it is a necessary mechanism which ensures that sufficient messenger reaches membrane translation sites from the gene. If hyperproduction of mRNA is indeed a reflection of the mechanism of enzyme secretion, it would explain continued secretion of depolymerase by *P. lemoignei* for nearly 30 min under conditions which are normally repressive.

To study the phenomenon further, we investigated the effect of rifampin on depolymerase secretion. If *P. lemoignei* accumulates depolymerase mRNA during the active secretion phase, it might be anticipated that a rifampin-insensitive phase of exoenzyme production would be observed after addition of the drug. Addition of rifampin before the onset of secretion completely inhibited enzyme secretion (at 30 and 60 min) (Fig. 8). However, its addition during the active secretion phase (at 90 and 120 min) did not prevent enzyme production for approximately 30 min. In this experiment, the pH at the time of addition of rifampin was 7.5 at 90 min and 7.7 at 120 min, and it was possible that the cells at the relatively high pH were incapable of immediate response to the drug. We therefore examined the effect of rifampin on secreting cultures maintained at a constant pH of 6.8 throughout the experiment (Fig. 9). Again

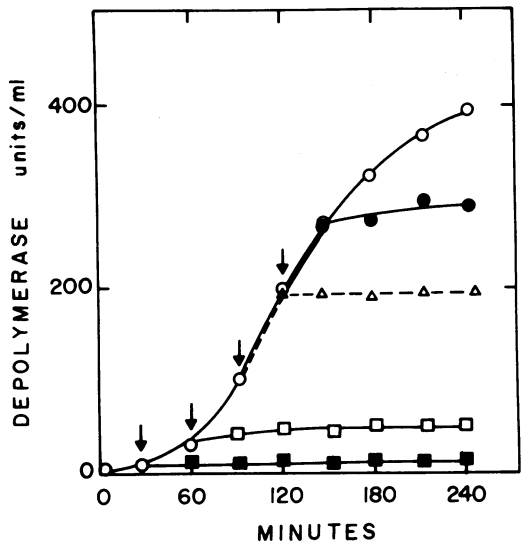

FIG. 8. *Effect of rifampin on depolymerase secretion. Rifampin was added at various time intervals to give a final concentration of 10 μg/ml. Rifampin additions: none (○); at 30 min (■); at 60 min (□); at 90 min (△); at 120 min (●).*

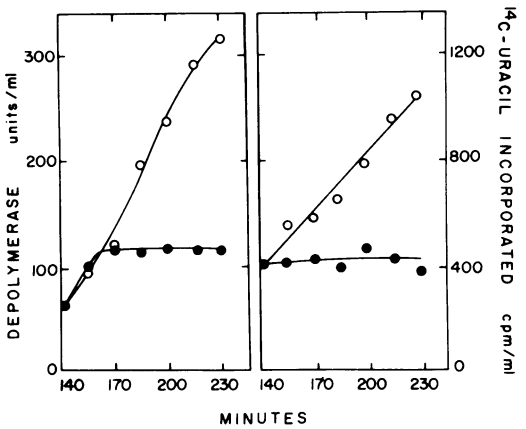

FIG. 9. *Effect of rifampin on depolymerase secretion and RNA synthesis. Washed cell suspensions were resuspended in secretion medium, and the pH was maintained at 6.8 throughout the experiment by the continuous addition of HCl. At 140 min, [2-^{14}C]uracil was added (0.8 μCi/ml) and the culture was divided into two equal suspensions, one of which received 30 μg of rifampin per ml. Rifampin addition: none (○); at 140 min (●).*

it was observed that secretion continued for nearly 30 min after addition of rifampin, whereas the incorporation of [^{14}C]uracil into trichloroacetic acid-precipitable material was immediately inhibited. Therefore, we conclude that synthesis and secretion of the exoenzyme had taken place in the absence of further mRNA synthesis.

Effect of inhibitors of protein synthesis on PHB depolymerase secretion. The synthesis of extracellular enzymes by gram-positive bacteria has been shown to be more susceptible to inhibitors of protein synthesis than general cell protein synthesis (2, 9). This has been interpreted as evidence for a membrane site for synthesis of extracellular enzymes. These sites, being more peripherally oriented than cytoplasmic sites, would be preferentially titrated by the inhibitor as it penetrates the cell, thereby exhibiting a greater sensitivity.

The sensitivity of PHB depolymerase secretion and total protein synthesis in *P. lemoignei* to inhibitors of protein synthesis acting at both the transcriptional (rifampin and streptovaricin) and translational (chloroamphenicol and puromycin) levels has been studied. Various concentrations of each inhibitor were added to cell suspensions containing L-[4,5-^{3}H]leucine. At appropriate intervals during incubation, samples of the suspension were removed and the amount of extracellular PHB depolymerase activity produced and the amount of [4,5-^{3}H]leucine incorporated into cell protein was determined. Table 1 shows the concentration of inhibitor required for 50% inhibition of extracellular enzyme secretion and general cell protein synthesis. In each case, a differential effect was noted; 5 to 10 times more inhibitor was necessary to stop general protein synthesis than synthesis of PHB depolymerase.

DISCUSSION

PHB depolymerase secretion by *P. lemoignei* is a typical example of the preferential production and elaboration of exoenzymes at the end of exponential growth. This phenomenon is well known (12, 22), but the mechanism of repression during the early growth phase and derepression in the stationary phase has not been satisfactorily explained. PHB depolymerase synthesis does not appear to be substrate induced, since this enzyme is made constitutively when *P. lemoignei* is grown on succinate or on other organic acids as sole carbon sources. Exoenzymes for the most part are hydrolases that act on large macromolecules (proteins, polysaccharides, nucleic acids), which cannot readily be transported into the cell to induce the synthesis of their appropriate metabolic enzymes. Thus, from a teleological point of view, repression of these scavenger enzymes during exponential growth on a preferred substrate and their synthesis in the absence of specific induc-

TABLE 1. *Effect of various inhibitors on enzyme secretion and cell protein synthesis*[a]

Inhibitor	Concn required for 50% inhibition (μg/ml)	
	Depolymerase secretion	[4,5-^{3}H]leucine incorporation
Chloramphenicol ..	0.90	5.0
Puromycin	5.00	55.0
Rifampin	0.35	1.9
Streptovaricin	12.00	83.0

[a] Varying amounts of chloramphenicol, puromycin, rifampin, and streptovaricin were added to washed cells resuspended in secretion medium containing [4,5-^{3}H]leucine. Samples (1 ml) of the cell suspension were taken at intervals, and the level of extracellular depolymerase and the incorporation of radioactivity into trichloroacetic acid-insoluble material determined as described in the text. Rates of enzyme secretion and protein synthesis were evaluated during the linear period between 60 and 180 min.

ers, after depletion of the substrate, has significant advantages in the economy and survival of the cell.

The most likely regulatory mechanism responsible for the prevention of exoenzyme synthesis by succinate is catabolite repression. There are, however, certain observations which make this conclusion still quite tentative. For example, attempts to reverse the repression by succinate with c-AMP or dibutyryl c-AMP were unsuccessful. Tanaka and Iuchi (30), in a study of the regulation of extracellular protease synthesis by *V. parahaemolyticus*, also observed that c-AMP was unable to overcome repression caused by glucose but did reverse glucose repression of the intracellular enzyme histidase. c-AMP has been shown to play an important role in the synthesis of a number of catabolic enzymes in *Escherichia coli* and other microorganisms and can reverse catabolite repression caused by glucose and other substrates (21). It may not, however, play a role in the synthesis of all inducible enzymes. For example, Rothman-Denes et al. (24) reported that c-AMP may not be essential for expression of the galactose operon.

Several investigators (2, 9, 17, 27) have proposed that the bacterial cell contains two sites for the synthesis of proteins. One such site could function for the synthesis of intracellular proteins, and the other, more peripherally located, (i.e., plasma membrane) would function in the synthesis of extracellular proteins. These conclusions were derived from studies carried out with protein synthesis inhibitors where it was shown that exoenzyme synthesis was considerably more sensitive to these inhibitors than was general protein synthesis. Similar effects with protein synthesis inhibitors were also observed on PHB depolymerase synthesis and cell-associated protein synthesis by *P. lemoignei*. Although it is tempting to interpret such data as support for a plasma membrane site for extracellular protein synthesis, a cautionary note must be raised. In all of the systems described, the exoenzymes are repressible. It has been observed that many catabolite-sensitive intracellular enzymes are preferentially repressed after exposure to certain antibiotics. Paigen (19) and Paigen and Williams (20) found that concentrations of chloramphenicol, which inhibit the growth rate by only 5%, produced almost complete repression of β-galactosidase. Similar effects were also observed with other antibiotics (20), Since it would appear that the differential inhibition observed in these studies was due to reasons other than separate sites for protein synthesis, no definitive conclusions can be drawn from the above experiments until the underlying regulatory mechanism for exoenzyme synthesis is understood.

Extracellular enzyme synthesis occurs in the absence of cell lysis and appears to be tightly coupled to secretion. The results obtained by others (1, 2, 9, 27) and those reported here support this conclusion. In addition to our studies with chloramphenicol, other efforts were carried out to establish the presence of accumulated cell-associated enzyme in *P. lemoignei*. It was noted earlier that *P. lemoignei* grows rapidly at pH 6.0 but secretes poorly; at pH 7.5 the reverse occurs. If enzyme is accumulated at the lower pH but not released because of an inactive release mechanism at the lower pH, then rapid shift of the cultures to pH 7.5 would result in a burst of accumulated enzyme appearing in the culture medium. All experiments of this type were negative. We also considered the possibility that depolymerase is accumulated during exponential growth and released during stationary phase as a result of lesions or alterations in the outer membrane of the cell evoked by nutritional deficiencies. There is, in fact, evidence which suggests that nutrient limitation can result in the release into the medium of outer-membrane components. Knox et al. (11) have shown that a lipopolysaccharide-phospholipid-protein complex is released by a lysine-requiring strain of *E. coli* during lysine deprivation. Rothfield and co-workers (13, 23) reported that increased release of outer-membrane complexes results upon cessation of pro-

tein synthesis. Thus, it is possible that the depolymerase is normally accumulated in the periplasmic space during active growth and is released during stationary phase along with outer-membrane complexes. Several attempts to detect the release of lipopolysaccharide during periods of maximal enzyme secretion were unsuccessful, indicating that such a mechanism as proposed above probably does not occur.

The dramatic effects of pH on enzyme secretion may be readily interpreted if we assume that the succinate transport system is pH sensitive; that is, at pH's greater than 7.0, succinate is transported too slowly to maintain a sufficient pool size of metabolic intermediates to support growth. When such conditions prevail, exoenzyme synthesis becomes induced. Attempts to repress exoenzyme secretion by rapidly lowering the pH has led to some interesting results. After decreasing the pH from 7.4 to 6.8, a time lag of 30 min was usually observed before repression of secretion was obtained. In view of our studies with chloramphenicol, it appeared unlikely that continued secretion under repressive conditions was due to preformed enzyme. Similarly, the immediate increase in the rate of uptake of succinate as well as its increased rate of conversion to trichloroacetic acid-insoluble substances at the lowered pH argued against the possibility that the delay was due to a time interval required to establish the repression (i.e., accumulation of sufficient quantities of a repressing effector). Further study of this effect demonstrated that there is a rifampin-insensitive phase of enzyme secretion and that this secretion (and presumably synthesis) was occurring in the absence of mRNA synthesis. Our results support the possibility that depolymerase mRNA accumulates during active secretion and is capable of supporting enzyme synthesis for up to 30 min under repressive conditions or in the presence of rifampin. They do not, however, rule out the alternative possibility that a stable mRNA is formed. In view of the studies of Both et al. (2) and Gould et al. (10), it would appear that the formation of an intrinsically stable messenger is unlikely. It would be of considerable interest to examine other bacterial enzyme-secreting systems to see whether mRNA accumulation is a general property of bacterial extracellular enzyme synthesis.

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