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Nature of *Escherichia coli* Cell Lysis by Culture Supernatants of *Bacillus* Species

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***Escherichia coli* cells were found to be sensitive to lysis by the supernatants of a variety of protease-positive *Bacillus* species when treated at 45°C but not when treated at 37°C. Different *E. coli* strains manifested different lysis sensitivities when treated at 45°C. When the lysis rates of *E. coli* cells at various stages of growth were investigated, post-exponential-phase cells were shown to be most sensitive to lysis. The lysis rate was inversely related to cell viability, and susceptibility appeared to be at least partly due to lysis of dead *E. coli* cells. A close relation was observed between levels of lysis activity and proteolytic activity. A *Bacillus subtilis* mutant lacking alkaline and neutral protease activity failed to lyse *E. coli* cells. It was concluded that *Bacillus* proteases played a major role in the observed *E. coli* lysis.**

The use of enzymes that lyse cell walls is a promising alternative to chemical disruption as a means of recovering intracellular microbial products (10, 13, 18, 20, 28, 36). The β-1,4-linkages between the *N*-acetylmuramic acid and *N*-acetylglucosamine residues of the peptidoglycan layer of bacterial gram-positive cell walls are hydrolyzed by egg white lysozyme, and this enzyme is widely used for lysis of gram-positive bacteria (26). Egg white lysozyme has been employed for large-scale isolation of catalase from *Micrococcus lysodeikticus* (5). Lysozymelike or other enzymes that lyse gram-positive bacteria are produced by *Agaricus bisporus* (8, 11), *Micromonospora* spp. (32), *Streptomyces coelicolor* (2), *Bacillus* spp. (21, 34), and a *Cytophaga* sp. (1). Previous studies in our laboratory led to the development of practical large-scale applications for using lytic enzymes to recover yeast protein (29).

Gram-negative bacteria are typically resistant to enzymatic lysis (3, 32). The peptidoglycan layer of gram-negative bacterial envelopes is protected from the action of lysozymelike enzymes by the outer membrane. Nevertheless, Pavlova et al. (23) reported that enzyme complexes that were capable of lysing vegetative cells of various gram-negative organisms were produced by a number of strains of thermophilic soil bacteria (23), and Fish et al. (9) used lysozyme to isolate alkane hydroxylase from *Pseudomonas putida*. Culture filtrates of *B. subtilis* YT25 contained a variety of enzymes such as proteases and bacterial lysozymes as well as an outer membrane-disruptive agent with properties similar to those of peptide antibiotics (19). In this case, it was concluded that the outer membrane is disrupted by a nonenzymatic factor, facilitating digestion of the cells by the enzymes (33). This complex lysed cells of *Pseudomonas aeruginosa* and *Escherichia coli*.

More recently, we have observed lysis of *E. coli* by *Bacillus* species on agar plates. We investigated the nature of this *E. coli* lysis to determine the lytic factors responsible and to evaluate any potential industrial utility.

MATERIALS AND METHODS

Preparation of *E. coli* substrate for lysis studies. Unless otherwise stated, the *E. coli* strain used in standard procedures was ATCC 23717. *E. coli* cells, obtained from nutrient agar stock cultures incubated at 37°C, were inoculated with a sterile wire loop into culture tubes containing 5 ml of nutrient broth (Difco) and incubated for 12 h at the production temperature. Aliquots of 1 ml of the resultant culture were inoculated into 250-ml Erlenmeyer flasks containing 100 ml of nutrient broth and incubated at 37 or 45°C on an orbital incubator shaker set at 200 rpm.

In the agar plate lytic screening procedures, *E. coli* shake flask cultures were incubated at 37°C for 24 h. A 15-ml volume of this culture was mixed well with 400 ml of molten nutrient agar (55°C) and distributed in 10-ml aliquots into sterile petri dishes; separate sets were incubated at 37 and 45°C for 48 h. The plates became evenly hazy with *E. coli* growth during these incubations.

Screening of *Bacillus* species for *E. coli* lysis capability. For initial screening of *Bacillus* colonies, the above plates were overlaid with 8 ml of nutrient agar. The *Bacillus* colonies were inoculated onto this overlay with a sterile loop. The plates were then incubated for a further 24 h at their respective temperatures. For screening of *Bacillus* supernatants for the presence of lytic enzymes, the agar overlay step was omitted and standard 7-mm wells were bored into the plates with a sterile metal cork borer. *Bacillus* culture supernatants (48 h) were sterilized by passage through a 0.2-μm-pore-size syringe filter, and 30-μl samples were placed in the wells of the *E. coli* plates. The supernatants were allowed to absorb into the plates, which were then inverted and incubated for 24 h at their respective temperatures.

Cultivation of *Bacillus* species. The *Bacillus* liquid culture medium for production of cell supernatants containing lytic activity was the sporulation medium (pH 6.9) described by Pearson and Ward (24), which contained the following (in grams per liter): cane molasses (78% dry matter), 10.5; corn starch, 10; yeast extract (Difco, Detroit, Mich.), 7.5; $CaCO_3$, 1.0. This medium (90 ml per 250-ml Erlenmeyer flask) was inoculated and incubated at 45°C on an orbital incubator shaker set at 220 rpm. For screening of *Bacillus* species, flasks were inoculated with a sterile wire loop directly from nutrient agar plates after 24 h of incubation at 37°C. In later studies solely involving *Bacillus subtilis* NRRL 14206, the cultures on nutrient agar plates were used to inoculate culture tubes containing 5 ml of nutrient broth. After incu-

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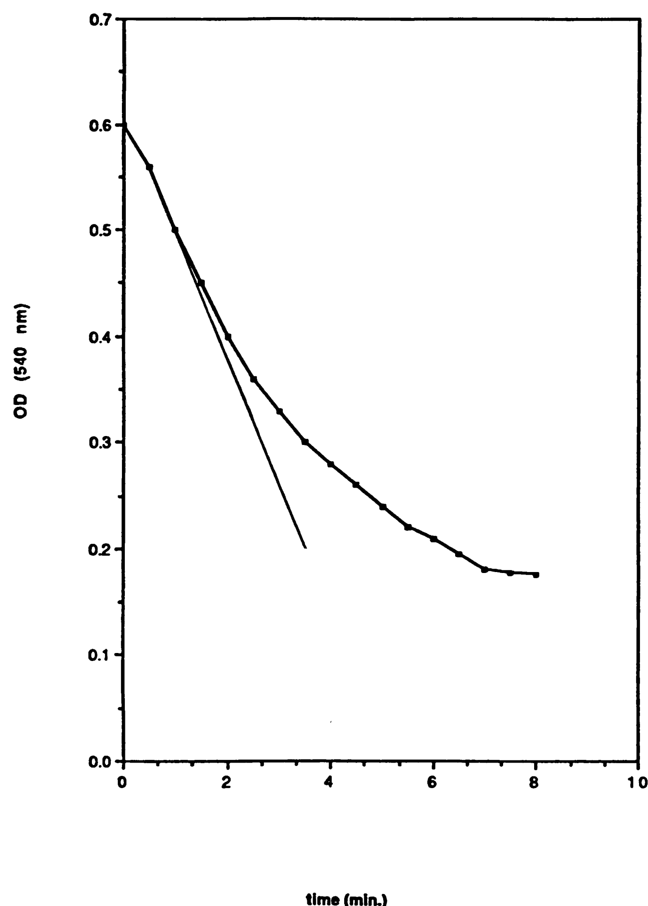

FIG. 1. Lysis of *E. coli* ATCC 23717 grown at 45°C for 72 h by *B. subtilis* NRRL 14206 cell supernatant. The straight line indicates the initial lysis rate.

bation of these tubes for 12 h at 45°C, 0.1 ml of the broth culture was used to inoculate each Erlenmeyer flask. *Bacillus* culture supernatants were prepared by centrifugation at 1,200 × *g* for 20 min at 4°C.

Spectrophotometric determination of lytic activity. Unless otherwise stated, 72-h *E. coli* shake flask cultures incubated at 45°C were harvested by centrifugation at 1,200 × *g* for 20 min, washed twice by suspension in fresh buffer (0.05 M Tris-HCl [pH 8.5]) at room temperature, centrifuged, and suspended in the same buffer to give an A_{540} of 1.2. A 1-ml volume of this cell suspension was mixed with 1 ml of *B. subtilis* culture supernatant in a cuvette with a 1-cm light path, and the decrease A_{540} was monitored (initial rates) by using a recording spectrophotometer (Shimadzu model UV-120-02) equipped with a temperature-controlled cuvette holder, set at 55°C unless otherwise stated. The slope ot the line indicating initial lysis rate was determined as illustrated in Fig. 1. Controls, prepared by the substitution of 1 ml of sterile *Bacillus* medium for the culture supernatant, manifested no decrease in A_{540}. One lysis unit is defined as a decrease in A_{540} of 0.001 per min under these conditions.

Determination of CFU. CFU were determined by using the standard pour plate counting method (4). Plates were incubated for 24 h at 37°C, and plates containing between 30 and 300 colonies were counted. Spore counts were determined by heating cells at 80°C for 10 min and then counting by the standard plate method.

Determination of protease activity. Zone clearance on skim milk-agar plates was used as a qualitative test for protease activity. Samples of 100 ml of 20% (wt/vol) skim milk powder and 100 ml of 2% (wt/vol) Difco technical agar were separately autoclaved at 121°C for 15 min, cooled to 55°C, and mixed together. After 15-ml aliquots of agar were dispensed into sterile petri dishes and allowed to set, 7-mm wells were formed in the plates; 30 μl of cell supernatant, sterilized by passage through a 0.20-μm-pore-size syringe filter, was applied to each well. The plates were incubated at 45°C for 12 h, and protease activity was determined by measuring zones of clearing on the plates.

Protease activity was quantitatively determined by measurement of trichloroacetic acid-soluble tyrosine after casein digestion. Hammarsten casein (0.5%, wt/vol) dissolved in 0.05 M Tris-HCl buffer (pH 8.0) was used as a substrate for protease determination. The enzyme solution (0.5 ml), appropriately diluted with Tris-HCl buffer (to ensure that the initial enzyme reaction rates were being measured), was added to 3 ml of substrate, and the mixture was incubated at 45°C for 30 min. The reaction was then terminated by the addition of 3 ml of 10% (wt/vol) trichloroacetic acid. Controls were prepared by adding the enzyme solution after the trichloroacetic acid precipitation step. Precipitated tube contents were filtered through two Whatman no. 1 filter papers, and the tyrosine content in 0.5 ml was assayed by the method of Lowry et al. (16).

Protease-negative *B. subtilis*. A *B. subtilis* double mutant deficient in extracellular alkaline and neutral proteases (strain DB104, genotype *his nprR2 nprE18 ΔaprA3*) was kindly provided by R. Doi (University of California, Davis). The strain carried lesions in the structural genes for extracellular neutral and serine proteases (15).

RESULTS

A range of *B. subtilis* strains and selected other *Bacillus* species were screened for lytic activity against *E. coli* ATCC 23717 by using the double-layer plate screening technique described in Materials and Methods. Two sets of plates were prepared; one set was incubated at 37°C, and one set was incubated at 45°C (Table 1). Plates incubated at 37°C showed no cell lysis. However, plates incubated at 45°C showed cell lysis by all *B. subtilis* strains and by *Bacillus licheniformis*, *B. pumilus*, and *B. megaterium*. Because the *Bacillus* colonies tended to spread on the surface of the agar plates to various degrees, measurement of zones of *E. coli* cell digestion for comparative purposes was not possible. Cell supernatants (after 48 h of culture) of *Bacillus* species grown in liquid culture media were tested for lytic activity against *E. coli* ATCC 23717 by using the plate screening method with incubations at 37 and 45°C. In addition, each supernatant was tested for protease activity by using a skim milk plate screening technique with incubations at 45°C (Table 1). The most active protease producers also manifested more limited protease zone clearing at 37°C. Again, no *E. coli* cell lysis was observed on plates incubated at 37°C, even after extended incubation times, indicating that the contrasting lysis data observed at 37 and 45°C were not due to simple kinetic differences. For plates incubated at 45°C, supernatants of *B. subtilis* NRRL 14206 manifested the largest zone of cell digestion. Supernatants of all of the strains that showed lytic activity against *E. coli* also exhibited protease activity. Supernatants of *B. megaterium* ATCC 14581, *Bacillus brevis* ATCC 8186, and *B. subtilis* NRRL 447 were both lysis negative and protease negative. Among the 16

TABLE 1. Screening of *Bacillus* colonies for lytic activity against *E. coli* ATCC 23717[a]

Organism	*E. coli* cell lysis[b]	Diameter (mm) of zone of *E. coli* cell lysis[c]	Protease activity[d]
B. brevis ATCC 8186	−	0	−
B. coagulans ATCC 7050	−	0	+
B. licheniformis ATCC 14580	++	20	+
B. megaterium ATCC 14581	+	0	−
B. polymyxa ATCC 842	−	8	+
B. pumilis ATCC 72	++	21	+
B. subtilis			
ATCC 6051	++	10	+
ATCC 6633	++	19	+
ATCC 9943	++	20	+
ATCC 21331	++	16	+
ATCC 21394	++	20	+
NRRL 207	++	18	+
NRRL 356	++	15	+
NRRL 447	++	0	−
NRRL 744a	++	16	+
NRRL 3544	++	16	+
NRRL 14206	+++	29	+
NRRL 14393	++	25	+
NRRL 14807	+++	18	+

[a] Lysis of *E. coli* cells did not occur at 37°C.

[b] *E. coli* cells were mixed in molten agar and incubated in petri plates at 45°C for 48 h. The plates were overlaid with nutrient agar, inoculated with *Bacillus* colonies, and incubated for 24 h. The plates were then examined for the presence of zones of *E. coli* cell lysis: −, no zone; +, very small zone; ++, large zone; +++, very large, clear zone.

[c] The overlay step was omitted; filter sterilized supernatants from 48-h *Bacillus* cultures (30 μl) were added to 7-mm wells bored in the agar, and the plates were incubated for 24 h.

[d] The same filter-sterilized culture supernatants (30 μl) were applied to 7-mm wells bored in skim milk agar, and the plates were incubated for 12 h. In this case, zone clearing was only expressed qualitatively because the agar had started to dry out, a problem not encountered in the thicker double-layer plates.

protease-positive supernatants tested, only the *Bacillus coagulans* ATCC 7050 culture supernatant was lysis negative. Two strains, *B. licheniformis* ATCC 14580 and *B. subtilis* NRRL 447, manifested zones of *E. coli* cell lysis around *Bacillus* colonies in overlaid agar plates, but the supernatants prepared from these strains did not exhibit lytic or proteolytic activity. Nevertheless, the results overall suggested a positive role for protease in the cell lysis observed. In addition, since the same *Bacillus* supernatants were used for both the 37 and 45°C lysis screening procedures, it appeared that the higher incubation temperature of 45°C rendered the *E. coli* cells susceptible to lysis.

A selection of *Bacillus* species cell supernatants giving large zones of lysis were used to test the relative susceptibilities to lysis of a range of *E. coli* strains incubated at 37 or 45°C (Table 2). None of the strains tested was susceptible to lysis when incubated at 37°C. At 45°C, all strains were susceptible to lysis by at least four of the six *Bacillus* supernatants. *E. coli* ATCC 10978 and ATCC 23227 appeared less susceptible to lysis by manifesting smaller zones of digestion with the supernatant of one *Bacillus* strain and relatively faint zones with supernatants of a number of other strains.

A spectrophotometric procedure was developed to measure the decrease in the A_{540} of *E. coli* ATCC 23717 cells due to lytic enzyme (see Materials and Methods). A typical lysis curve is presented in Fig. 1. The effect of *E. coli* cell viability

TABLE 2. Relative susceptibilities of *E. coli* strains to lysis by six *Bacillus* strains

Bacillus strain	Diameter (mm) of zone of cell digestion for *E. coli* strain				
	ATCC 23176	ATCC 23717	ATCC 9323e	ATCC 10978	ATCC 23227
B. licheniformis ATCC 14580	24	23.5	22	23.5[a]	23
B. pumilus ATCC 72	13.5	14	15	13[a]	14[a]
B. subtilis NRRL 207	14.5	14	14		14[a]
B. subtilis ATCC 6633	10.5	11	10		
B. subtilis NRRL 14206	25	25	25	25[a]	25
B. subtilis NRRL 14393	23	23	23	23.5	23

[a] Faint zone.

on lysis susceptibility was investigated by using *E. coli* cells recovered after various time intervals from nutrient broth cultures grown at 37 and 45°C (Fig. 2). *E. coli* cells grown at 37°C showed relatively slow reduction in viability after the active growth stage and manifested very low lysis susceptibility. Lysis susceptibility, in contrast with that in *E. coli* grown at 45°C, appeared to be at least partly related to cell death. However, since the number of nonviable *E. coli* cells increases at an approximately exponential rate, whereas the increase in the lysis rate is linear, other factors may be important.

Since protease was present in all of the supernatants

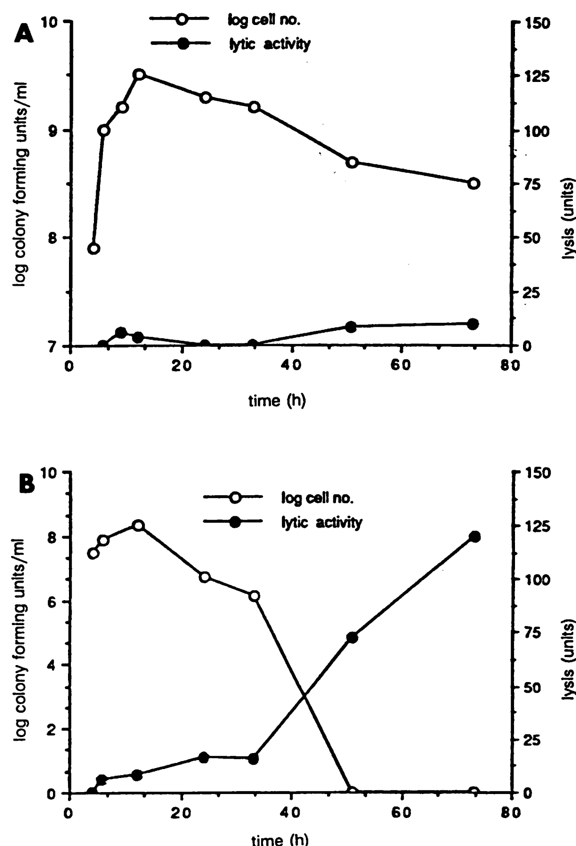

FIG. 2. Relationship between viability of *E. coli* ATCC 23717 grown at 37°C (A) and 45°C (B) and susceptibility to lysis by *B. subtilis* NRRL 14206 supernatant.

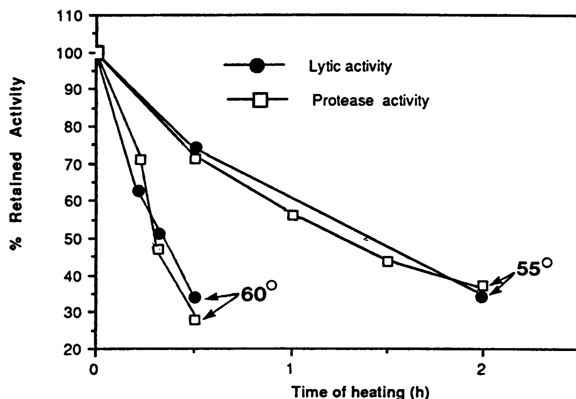

FIG. 3. Comparison of thermal inactivation of *B. subtilis* NRRL 14206 protease and lytic enzyme at 55 and 60°C.

exhibiting lytic activity, experiments were conducted to further investigate the relationship between *E. coli* lysis by *B. subtilis* NRRL 14206 and protease activity. The pattern of production of protease and lysis of *E. coli*, the percentage of sporulated cells, and the culture pH were monitored for *B. subtilis* NRRL 14206 grown in liquid culture at 45°C under standard conditions. Protease levels over time in the culture supernatant corresponded very closely with lytic activity against *E. coli* (data not shown). Culture supernatants (96 h) from *B. subtilis* NRRL 14206 were incubated at 55 and 60°C, and the patterns of thermal inactivation of lytic activity and protease activity were monitored as a function of time (Fig. 3). A close parallel was observed between the percentages of retained lytic and protease activities at both temperatures. The effect of pH on lytic activity against *E. coli* was determined by using the spectrophotometric lysis assay. The pH optimum for lysis was pH 8.5 through 9; greater than 50% of optical lysis activity was observed at pH 5.5 through 9.5. This pH activity profile is typical of *B. subtilis* alkaline proteases. The relationship between lytic and protease activities in supernatants of *B. subtilis* NRRL 14206 and for a commercially purified and crystallized *B. subtilis* alkaline protease (type VIII; Sigma, St. Louis, Mo.) are compared in Fig. 4. The results confirm that purified alkaline protease from *B. subtilis* lyses *E. coli*, suggesting that protease plays a major role in the cell lysis observed with the *B. subtilis* NRRL 14206 supernatant. It should be noted that the ratio of lytic activity associated with the protease of *B. subtilis* NRRL 14206 strain supernatant to protease activity is greater than that of the commercial purified alkaline protease. These analyses were the averages of three determinations and were reproducible. Thus, the differences observed between the lytic activities of the *B. subtilis* supernatant and the purified enzyme are considered to be significant.

Although the above data suggested a role for proteases in the lysis of *E. coli*, they were not at all conclusive. *E. coli* lysis by *B. subtilis* ATCC 12406 could be inactivated by boiling the supernatant, thereby indicating that the lytic factor manifested the normal heat sensitivity observed for enzymes and proteins. The two major proteases produced extracellularly by *Bacillus* species are the neutral metalloprotease and the serine alkaline protease. A well-characterized protease-negative *B. subtilis* mutant was therefore examined for lytic activity against *E. coli*. The experiments documented in Tables 1 and 2 were repeated with a *B. subtilis* protease-negative mutant that lacked both extracellular alkaline and neutral proteases (strain DB104) (14). Neither colonies nor cell supernatants of *B. subtilis* DB104 lysed *E. coli* ATCC 23717 at 37 or 45°C with the agar plate screening methods. The inability of supernatants from the protease-negative mutant of *B. subtilis* to lyse *E. coli* cells therefore provides the strongest supporting evidence for the role of *Bacillus* proteases in the lysis of *E. coli*.

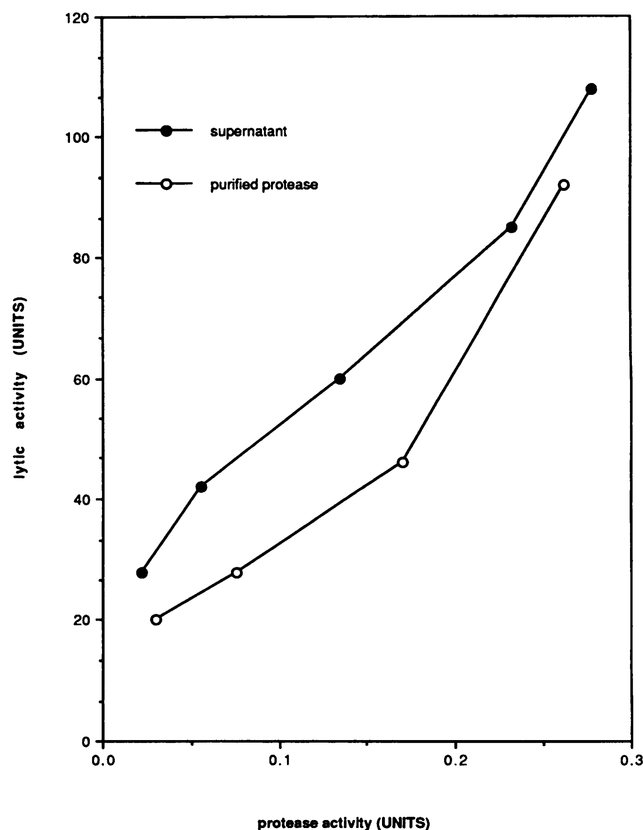

FIG. 4. Relationship between protease activity and lytic activity. Supernatant from a 96-h shake-flask culture of *B. subtilis* NRRL 14206 was diluted with 0.05 M Tris-HCl buffer (pH 8.0) to give a series of protease activities in the range of 0.02 to 0.28 U/ml). Solutions of commercial *B. subtilis* alkaline protease (Sigma) were prepared in a similar manner. These solutions were assayed for lytic activity against *E. coli* ATCC 23717 by using the spectrophotometric method.

DISCUSSION

The research results presented in this paper indicate that proteases produced by *Bacillus* strains are major factors responsible for the observed lysis of *E. coli* cells. The observation that a mutant strain of *B. subtilis*, which lacked both extracellular alkaline and neutral proteases, failed to lyse *E. coli* cells provided the most compelling evidence of the lytic role of these *Bacillus* proteases. In addition, results suggest that post-log-phase nonviable cells or dying cells, produced by incubation at 45°C, may be particularly sensitive to cell lysis (at least for these short reaction times). A perfect correlation between *Bacillus* lytic activity and the presence of protease was not observed. *B. coagulans* manifested proteolytic activity but not lytic activity. However, *B. coagulans* proteolytic activity appears to be different

from those of *B. subtilis* and the other protease-positive, lysis-positive strains (*B. licheniformis*, *Bacillus polymyxa*, and *B. pumilus*). All the latter strains are characterized as hydrolyzing casein and gelatin. Although some *B. coagulans* strains hydrolyze casein, *B. coagulans* strains do not hydrolyze gelatin (31). It was also noted that, although *B. licheniformis* ATCC 14580 and *B. subtilis* NRRL 445 lysed *E. coli* cells in overlaid agar plates, their supernatants had neither lytic nor protease activity. The *E. coli* lysis observed on overlaid plates may have been caused by intracellular proteases from these *Bacillus* strains released as a result of *Bacillus* cell autolysis. The induction of enzyme lysis by heat treatment of *E. coli* has been previously indicated by Desai and Dhala (7), who reported that a lytic protease produced by thermophilic actinomycetes lysed *E. coli* cells, and by Fermor and Wood (8), who observed lysis of autoclaved *E. coli* cells by a lytic enzyme complex produced by *Agaricus bisporus*.

Heat treatment of cells can result in substantial damage, with release of outer membrane lipopolysaccharides, protein release, blebbing and vesiculation of the cell surface, and leakage of periplasmic enzymes (14, 17, 35). The blebbing may be due to disruption of the lipoprotein bonds anchoring the outer membrane to the underlying peptidoglycan, since lipoprotein-deficient mutants also exhibit blebbing (22). Divalent cations (such as magnesium) that are important for lipopolysaccharide interactions increase the resistance of the cell envelope to heat treatment, whereas chelating agents enhance the disrupting effects of heat on the outer membrane of *E. coli* (12, 35). The peptidoglycan layer of *E. coli* appears to be relatively heat stable and is virtually unaffected by temperatures of up to approximately 48°C (12).

From the present study, the susceptibility of post-exponential-phase *E. coli* cells incubated at 45°C to lysis by *Bacillus* culture filtrates appears to result from heat disruption of the outer cell membrane (12, 35) and the resultant exposure and hydrolysis of the peptidoglycan layer by *Bacillus* protease. Chemical methods for disrupting the outer cell membrane include the use of EDTA or polymyxin, which also render *E. coli* cells susceptible to enzymatic lysis by proteases, phospholipase, or lysozyme (19, 25, 27, 30, 33). When these chemicals are used to disrupt the outer membrane, enzymatic lysis can be observed, even at room temperature (6). The slightly higher ratio of lytic activity to protease activity of *Bacillus* culture supernatants compared with that of purified *Bacillus* alkaline protease may indicate the presence of other nonprotease enzymes contributing to cell lysis by the *B. subtilis* culture supernatants. The variations in susceptibility of different *E. coli* strains may be due to differences in the outer membrane or peptidoglycan composition.

The use of a combination of protease and mild heat treatment may have applications for the recovery of nonpeptide molecules from gram-negative microorganisms.

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