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Hyperprotease-Producing Mutants of *Bacillus subtilis*

THOMAS B. HIGERD, JAMES A. HOCH, AND JOHN SPIZIZEN

Department of Microbiology, Scripps Clinic and Research Foundation, La Jolla, California 92037

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A number of mutants of *Bacillus subtilis* producing high levels of extracellular protease have been isolated. Analysis of culture supernatants of these mutants has shown that the total amount of proteolytic activity is elevated from 16- to 37-fold over the wild strain. The elevated activity was due to a simultaneous increase in both the neutral and alkaline protease. All of the mutants genetically analyzed were found linked to the *argC4* marker by PBS-1 transduction analysis.

The existence of at least three distinct proteolytic enzymes has been well established in sporulating cultures of *Bacillus subtilis*. One of the enzymes is a casein-hydrolyzing alkaline protease belonging to the subtilisin class of endopeptidases (4, 12, 15). The second enzyme, neutral protease, containing zinc as the prosthetic group (10) is one of the most active casein-hydrolyzing enzymes known and is readily inactivated by chelating agents. A third protease has been reported which does not attack casein appreciably but possesses high esterase activity (2, 13, 14).

The increase in extracellular proteolytic activity in *Bacilli* undergoing sporogenesis has suggested a correlation between protease production and spore formation (6, 7, 11). This association is strengthened by the fact that certain mutants that are unable to hydrolyze casein are also asporogenous (9, 16). Mutants defective in certain proteases have been reported to sporulate normally (17, 13). In order to clarify this relationship, a search for specific protease-defective mutants of *B. subtilis* 168 was initiated. In the process, several mutants were found that produced an abnormally large zone of hydrolysis on casein plates. This report concerns the determination of the two casein-hydrolyzing activities in mutant and wild-type strains and the genetic mapping studies of the mutants.

The strains of *B. subtilis* used in this study were: W168 (prototroph), 168 (*trpC2*), GSY 712 (*metC1*), and BR 85 (*trpC2, argC4*). Hpr (hyperprotease-producing) strains were obtained by mutagenizing strain 168 spores with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1) and ethyl methane sulfonate (2). Mutagenized spores were plated on the medium described below, and clones exhibiting large halos of casein hydrolysis were picked and purified for further study.

The solid growth medium found most suitable for high protease titers consisted of: 1.0% glucose, 0.5% casein (Difco), 0.5% yeast extract (Difco), Spizizen's minimal salts (18), and 1.5% Noble agar. The entire agar surface was inoculated with approximately 5×10^7 cells per plate. After incubating for 48 hr at 37 C, the petri dishes were stored frozen at −70 C. After 18 hr, the agar preparations were allowed to thaw at 4 C and were centrifuged. The cell-free supernatant fluid was then dialyzed overnight against cold 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, *p*H 7.5, and subsequently used in the amylase and protease assays.

The saccharogenic assay of Fischer and Stein (3) as modified by Welker and Campbell (19) was used to measure amylase activity. One unit of activity was defined as that amount of enzyme which will release 1 mg of reducing groups, standardized to maltose, in 3 min at 25 C, *p*H 6.3.

A modification of Hagihara's method (5) was employed to measure protease units. The reaction mixture contained 0.5 ml of enzyme solution, 0.5 ml of 0.05 M Tris-hydrochloride buffer, *p*H 7.5, and 5.0 ml of 1.2% Hammersten's casein (Mann Research Lab) dissolved in the same buffer. In all cases, tubes containing 1.0 ml of enzyme solution and buffer were preincubated for 10 min at 30 C prior to substrate addition. Incubation of the reaction mixture was performed at 30 C for 10 min. One unit of protease activity was defined as that amount of enzyme which liberates trichloroacetic acid-

soluble products giving an extinction at 275 nm equivalent to 1 μg of tyrosine in 1 min at 30 C.

Since the neutral protease contains a zinc prosthetic group, it is readily inactivated by chelating agents (10). A titration with ethylenediaminetetraacetic acid (EDTA) of a supernatant fluid from a W168 culture is shown in Fig. 1. The total proteolytic activity decreases with increasing EDTA concentration until a constant value is obtained. The difference between the initial value and the EDTA-insensitive value is taken to be the contribution of the neutral protease to the total proteolytic activity. Since the third protease that has been found does not attack casein appreciably, we assume the residual activity is mostly the alkaline protease. In all subsequent experiments, the relative contribution of the two proteases to the total proteolytic activity was determined in the presence or absence of 8×10^{-3} M EDTA.

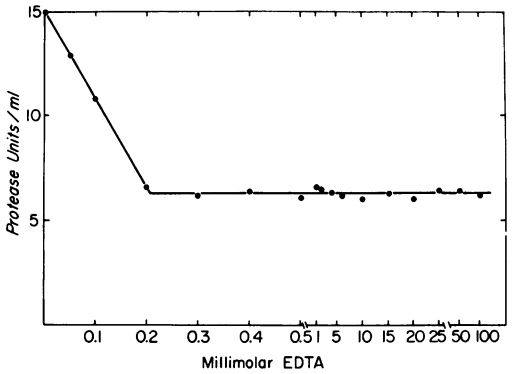

FIG. 1. *Proteolytic activity of W168 culture supernatant fluid preincubated with various concentrations of EDTA.*

TABLE 1. *Protease activity of W168 and the high protease producers (hpr)*

Strain	Amylase units/ml	Protease units/ml		Per cent activity assigned to:	
		No EDTA	EDTA	Neutral protease	Alkaline protease
W168	0.41	15	6.3	58	42
hpr 1	0.36	484	101	79	21
hpr 2	0.36	476	96	80	20
hpr 3	0.40	523	115	78	22
hpr 4	0.38	491	101	79	21
hpr 5	0.36	505	112	78	22
hpr 6	0.41	495	121	76	24
hpr 7	0.42	504	100	80	20
hpr 8	0.38	466	84	82	18
hpr 9	0.36	455	87	81	19
hpr 10	0.37	557	115	79	21
hpr 11	0.46	515	104	80	20
hpr 12	0.40	542	113	79	21
hpr 13	0.45	499	102	80	20
hpr 14	0.37	465	95	80	20
hpr 15	0.39	504	95	81	19
hpr 16	0.38	446	88	80	20
hpr 17	0.37	504	57	89	11
hpr 18	0.40	248	33	87	13

In Table 1 is shown the protease activity of W168 and 18 high protease-producing (hpr) mutants isolated on casein plates described above. As a control for an additional extracellular enzyme, the amount of amylase produced by each strain was determined. It can be seen that the total amount of amylase is not significantly different in any of the high protease-producing strains from the wild type. However, the total amount of protease varies from 16- to 37-fold higher in the 18 mutants, with an average value of 33-fold higher than the wild type. Notice also that there is a slight shift in the relative proportions of neutral and alkaline proteases. In the mutants, the neutral contribution is about 80% of the total proteolytic activity, whereas in the wild type it is about 60%. It also can be seen that both the neutral and the alkaline proteases are increased in these hyper-protease-producing strains.

PBS-1 transducing lysates were prepared with the mutant strains as described previously (8). In two-factor crosses with *argC4* and *metC1* recipients (Table 2), all of the hpr mutations were found linked to these two markers with about the same recombination values as were

TABLE 2. *Linkage of high protease-producing (hpr) mutations with argC4 and metC1 in two-factor PBS-1 transduction crosses*

Donor	Recipient (genotype)	hpr/ *met*$^+$	Per cent recombination	Recipient (genotype)	hpr/ *arg*$^+$	Per cent recombination
hpr 1	*metC1*	0/80		*argC4*	21/100	79
hpr 2	*metC1*	6/80	92	*argC4*	32/100	68
hpr 5	*metC1*	4/80	95	*argC4*	22/100	78
hpr 6	*metC1*	2/80	97	*argC4*	19/100	81
hpr 7	*metC1*	4/80	95	*argC4*	27/100	73
hpr 8	*metC1*	5/80	94	*argC4*	20/100	80
hpr 9	*metC1*	3/80	96	*argC4*	13/100	87
hpr 10	*metC1*	2/60	97	*argC4*	6/100	94
hpr 11	*metC1*	8/80	90	*argC4*	16/100	84
hpr 12	*metC1*	5/80	94	*argC4*	21/100	79
hpr 13	*metC1*	5/60	92	*argC4*	14/87	84
hpr 14	*metC1*	4/40	90	*argC4*	15/100	85
hpr 15	*metC1*	1/17	94	*argC4*	9/75	88
hpr 16	*metC1*	2/19	90	*argC4*	14/51	73
hpr 17	*metC1*	1/16	94	*argC4*	9/75	88
hpr 18	*metC1*	1/20	95	*argC4*	20/100	80

found previously for the hpr-97 marker (9). Two mutants, hpr 3 and hpr 4, are not included in this analysis since we were unable to prepare PBS-1 lysates on either of the mutants. Thus, of the mutants in which we were able to do genetic analysis, all appear to lie closer to *argC4* than to *metC1*. The *argC4* and *metC1* markers are separated by about 80% recombination in PBS-1 transduction analysis.

These results indicate that the majority of the high protease-producing mutants contain mutations in the *argC4* region. The biochemical basis for this hyperproduction is still unknown.

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