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Initial studies of the use of aminopeptidases for the differentiation of *Bacillus sphaericus* strains

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SUMMARY

Analysis of aminopeptidase activity in commercially available enzyme panels (SAP-II and SAP-III) indicated qualitative differences among the eight strains of *Bacillus sphaericus* tested. These differences involved specific substrates utilized, the importance of sampling time, as well as differences between whole cells and cell-free extracts. Further detailed analysis of four of the L-amino acid-β-naphthylamide substrates using polyacrylamide gel electrophoresis (PAGE) demonstrated a series of allelic differences in the multilocus patterns observed. The resulting phenogram constructed from the enzyme and PAGE information showed a marked separation of the noninsecticidal strains from the insecticidal strains (58% similarity). In addition, there was a separation within the insecticidal strains of strain SSII-1, phage type 2 from strain 2297, phage type 4, and of both of these strains from the remaining highly insecticidal members of phage type 3 (strains 1593, 2013-4, 2362, 2500).

INTRODUCTION

Since the initial isolation of insecticidal strains of *Bacillus sphaericus* [10,17], it has been apparent that there are few easily detectable phenotypic differences between the insecticidal and noninsecticidal strains [18]. This has been confirmed and well-documented [3,5,24]. Differences between insecticidal and noninsecticidal strains were distinguished using a numerical analysis of results from auxanograms of 160 substrates [6]. A more valuable approach was serotyping by means of flagellar antigens [6]. Similarly, these strains were differentiated using bacteriophage typing [25]. Utilizing DNA homology [11], 67 strains of *B. sphaericus* were grouped into five homology groups (with group II divided into two subgroups; see also Refs. 13 and 15). None of the above schemes is completely satisfactory, however, in distinguishing among insecticidal strains. A more modern biochemical systematics approach was suggested by Schleifer and Stackebrandt [14], who said that at the epigenetic level, analysis of homologous proteins via functional studies (zymograms) is an excellent and reliable way to differentiate closely related strains of a spe-

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cies as well as closely related species. The purpose of this study was to differentiate phenotypically between insecticidal and noninsecticidal strains of *B. sphaericus* by examining qualitatively the aminopeptidase substrate specificity patterns using 24 L-amino acid-β-naphthylamide substrates for aminopeptidase activity. Subsequently a detailed analysis was performed on four of these substrates using polyacrylamide gel electrophoresis in the hope of finding differences in electromorph patterns among the strains.

MATERIALS AND METHODS

Organisms and growth conditions

Noninsecticidal *B. sphaericus* strains 14577 and 7054 were each derived from their ATCC counterpart. Insecticidal *B. sphaericus* strains 1593, 2297, 2500, 2362, SSII-1, 2013-4 were each derived from WHO/CCBC accession counterparts (with SSII-1 being derived from WHO/CCBC accession No. 1321). Cultures were maintained on BHI^+ agar (brain heart infusion agar plus vitamins including, per l: thiamine-HCl, 10 mg; nicotinic acid, 10 mg; Ca pantothenate, 10 mg; biotin, 1 mg) and on YNB^+ (nutrient agar supplemented with 0.05% yeast extract plus 1×10^{-3} M $MgCl_2$, 7×10^{-4} M $CaCl_2$, 5×10^{-5} M $MnCl_2$) plus vitamins.

A standard inoculum buildup was performed [19] in which a loopful from a 2-day-old fresh slant was used to inoculate a roller tube containing 5 ml of YNB^+ broth. This 5 ml culture was incubated on a roller drum apparatus at 26 rpm for 16 h at room temperature. The inoculum was then transferred to a flask containing 25 ml of YNB^+ in a 125-ml Erlenmeyer flask which was incubated at 30°C in a gyrotory shaker at 300 rpm. Culture material was taken at 3, 12, or 20 h depending upon the experimental protocol needs.

Preparation of bacterial samples

Whole cells were used for the taxonozyme panels, unless otherwise stated. Culture material for the panels was centrifuged and washed twice and the cells resuspended in 0.1 M Tris, pH 8.5 buffer at 2700 × *g*. Cultures were adjusted to a McFarland No. 5 nephelometer standard and 50 μl were used to inoculate each well. For the preparation of bacterial cell-free extracts, a 75-ml culture sample was centrifuged at 2700 × *g* and the bacterial cells were washed twice with 30 ml of cold 0.1 M Tris/40 mM EDTA · Na_2, pH 8.5. Bacterial cells were then resuspended in 10 ml of the buffer and passed through a French Pressure Cell at 12000 psi. This material was centrifuged at 5900 × *g* and a Lowry protein determination was made of the supernatant material which was adjusted to 1 mg protein/ml and stored in 0.5 ml aliquots at −20°C to be used within 3 weeks.

Chemicals and reagents

Bacterial enzyme activity was scanned by using commercially available aminopeptidase SAP-II and SAP-III Taxonozyme panels (EY Laboratories, San Mateo, CA). According to the manufacturer, these contained all the necessary substrates and cofactors. Substrates found in each panel (and their abbreviations) were, for SAP-II: alanyl-β-naphthylamide (Ala-β-NA); arginyl-β-naphthylamide (Arg-β-NA); cystyl-β-naphthylamide (Cys-β-NA); γ-glutamyl-β-naphthylamide (γ-Glu-β-NA); glycyl-β-naphthylamide (Gly-β-NA); leucyl-β-naphthylamide (Leu-β-NA); lysyl-β-naphthylamide (Lys-β-NA); phenylalanyl-β-naphthylamide (Phe-β-NA); prolyl-β-naphthylamide (Pro-β-NA); glutamylphenylalanyl-β-naphthylamide (Glu-Phe-β-NA); valylalanyl-β-naphthylamide (Val-Ala-β-NA); alanylalanylphenylalanyl-β-naphthylamide (Ala-Ala-Phe-β-NA); for SAP-III: histidyl-β-naphthylamide (His-β-NA); hydroxyprolyl-β-naphthylamide (OH-Pro-β-NA); seryl-β-naphthylamide (Ser-β-NA); tyrosyl-β-naphthylamide (Tyr-β-NA); tryptophanyl-β-naphthylamide (Trp-β-NA); valyl-β-naphthylamide (Val-β-NA); glycylglycyl-β-naphthylamide (Gly-Gly-β-NA); glycylphenylalanyl-β-naphthylamide (Gly-Phe-β-NA); leucylglycyl-β-naphthylamide (Leu-Gly-β-NA); seryltyrosyl-β-naphthylamide (Ser-Tyr-β-NA).

Aminopeptidase SAP-II panel substrates were stable and could be used to demonstrate hydrolysis of substrate at various time periods. Substrates in

the aminopeptidase SAP-III class were less stable and had to be tested within 4 h; therefore, they did not allow testing for extended periods of time. For the polyacrylamide gel electrophoresis (PAGE), the following L-amino acid-β-naphthylamide substrates (Sigma) were used for the detection of enzyme activity: L-alanine-β-naphthylamide crystalline free base (Ala-β-NA); L-leucine-β-naphthylamide, crystalline free base (Leu-β-NA); L-serine-β-naphthylamide, crystalline free base (Ser-β-NA); glycyl-L-phenylalanine-β-naphthylamide, crystalline free base (Gly-Phe-β-NA).

Taxonozyme panel method and analysis

The procedure for the taxonozyme panels was based on the recommended use by the manufacturer of the panel (EY Laboratories) and adapted to the requirements of each panel class. This consisted of adding 50 μl of whole cell preparation (or an equivalent volume of cell-free preparation) into each well, incubating at 37°C and examining as follows. Aminopeptidase SAP-II panels were scored at 0.5, 1, 2 and 16 h for a positive yellow color reaction.The aminopeptidase SAP-III panel was checked within 4 h, at which time one drop of a reconstituted EY-20 dye (EY Laboratories) was added to each well and a positive red color reaction noted. Substrate activity for each of the panels was determined by the presence or absence of enzymatic hydrolysis of each substrate as described above.

Polyacrylamide gel electrophoresis (PAGE)

The PAGE procedure was a modification of the alternative method used by Davis [4]. A 7% separating gel and 3% stacking gel were used. Thirty μl of the cell free extracts was loaded into each well. Twenty microliters of 50% sucrose 0.0001% bromophenol blue was added to each well and gently mixed. A continuous running buffer system (Tris-glycine, pH 8.3) was used. Gels were run at 5–10°C and a constant current of 10 mA per gel was applied until the tracking dye just entered the stacking gel. Current was then increased to 30 mA per gel until the tracking dye was 10 mm from the bottom of the gel. Each gel was then removed and stained. Optimum pH for enzyme activity was determined by splitting the gel into several sections and staining each portion at a different buffer pH. Optimum pH was then used in the staining procedure for the detection of enzyme activity in the strains tested. The optimum pH values were: Ala-β-NA, pH 7.0; Leu-β-NA, pH 7.5; Ser-β-NA, pH 7.0; Gly-Phe-β-NA, pH 7.5. Since the addition of cobalt (5 mM $CoCl_2$) enhanced enzyme activity, as represented by the number of electrophoretically detectable bands, cobalt was incubated with all the β-naphthylamide substrates used in PAGE. Bands were numbered consecutively with the most anodal band numbered '1'. Staining procedures for detection of enzyme hydrolysis of L-amino acid-β-naphthylamide substrates on PAGE were as follows. The gel was soaked in 5 mM $CoCl_2$ and incubated at 37°C while rotating at 105 rpm for 20 min. The gel was washed in 0.5 M Tris-borate buffer containing 5 mM $CoCl_2$ at the appropriate pH and then incubated at 37°C while being rotated as above. The buffer was discarded and 1 mg/ml of the β-naphthylamide substrate in 0.2 M Tris-malate buffer at the appropriate pH was added and the gel was incubated and rotated as above. Fast Garnet GBC salt (Sigma) adjusted to a final concentration of 0.5 mg/ml in 0.2 M Tris-malate buffer at appropriate pH was added and the gel was incubated and rotated as above until the red bands appeared. The gels were then rinsed in deionized water and examined and scored. The polyacrylamide gels were scored by dividing the migration distance of the band from the top of the separating gel to the center of the protein band by the migration distance of the bromophenol blue tracking dye from the top of the separating gel (R_f value).

The following definitions [1] may be useful for those unfamiliar with the terminology used in this report. Allele is used in the classical genetic sense, being one of two or more alternative forms of a gene, each possessing a unique nucleotide sequence. Protein variants controlled by allelic variants at a single gene locus and detected by electrophoresis are called allozymes or electromorphs. These are the 'bands' that one sees on the PAGE gel. The electrophoretic mobility of the 'band' (electromorph or allozyme) is represented by an R_f value.

In terms of the PAGE gel, a zone of bands is seen, which represents the area or region where the product of a specific gene locus is found. Each band represents an allele of that locus. Bands across the columns (within a specific lane) represent a single locus possibly containing several alleles. Alleles would be seen as slight differences in the R_f values across the zone of bands. In a multilocus situation as seen in this study, bands down each of the columns on a gel represent possibly different loci. Polymorphic refers to the presence of several forms (of a trait or of a gene) in a population. Monomorphic refers to the presence of a single form (of a trait or of a gene) in a population. The aminopeptidases in this study were all shown to be polymorphic. Several enzymes not reported here were monomorphic.

Statistical methods

The genetic relatedness among the eight *B. sphaericus* strains was estimated by making a phenogram from an unweighted-pair-group cluster-analysis with arithmetic averages (UPGMA) [20]. This was done by comparing percent similarities of three classes of characteristics [12]. The first characteristic was the percent of electrophoretic bands in common between each strain. The number of bands and R_f value for each band were used with each band representing one distinct characteristic. The bands were considered to be the same if the R_f values were within $\pm 5\%$ of each other. The second characteristic was enzyme activity, or the absence or presence of substrate hydrolysis. The third characteristic was the time for substrate hydrolysis to occur, with each enzymatically hydrolyzed substrate constituting a separate characteristic. The percent similarity between each strain was calculated by dividing the number of similar characteristics between two strains by the total number of characteristics studied.

RESULTS

Taxonozyme panels

Aminopeptidase activity of 3-h whole-cell preparations of eight strains of *B. sphaericus* (six insecticidal strains, SSII-1, 1593, 2013-4, 2297, 2500 and 2362; two noninsecticidal strains, 14577 and 7054)

Table 1

Summary of aminopeptidase substrates utilized by *B. sphaericus* strains

The results given are for a 3-h sampling time using whole cells.

Aminopeptidase panel[a]	Substrate utilized by all strains	Substrate utilized by some strains		Substrate not utilized by any strains
SAP-II	Ala-β-NA Arg-β-NA Leu-β-NA Pro-β-NA Glu-Phe-β-NA Ala-Ala-Phe-β-NA	Lys-β-NA Val-Ala-β-NA	(all but SSII-1)	Cys-β-NA γ-Gly-β-NA Phe-β-NA Gly-β-NA[b]
SAP-III	Met-β-NA Ser-β-NA Gly-Phe-β-NA	Tyr-β-NA Gly-Gly-β-NA Leu-Gly-β-NA	(only by 14577, 7054)	His-β-NA OH-Pro-β-NA Ile-β-NA Trp-β-NA Val-β-NA Ser-Tyr-β-NA

[a] Aminopeptidase panels (E-Y Laboratories, San Mateo, CA).

[b] Gly-β-NA substrate autohydrolyzed each time used.

grown on YNB$^+$ medium, was examined using commercially available SAP-II and SAP-III aminopeptidase substrate panels (Table 1). Seven substrates from the SAP-II panel and three substrates from the SAP-III panels were utilized by all of the strains tested, while four substrates from the SAP-II panel and six substrates from the SAP-III panel were not utilized by any of the strains. Five substrates, Lys-β-NA, Val-Ala-β-NA (from SAP-II) and Tyr-β-NA, Gly-Gly-β-NA and Leu-Gly-β-NA (from SAP-III), were utilized by only some of the strains (Table 1).

The effect of sampling time on aminopeptidase (SAP-III) enzyme activity in whole-cell preparations and cell-free extract preparations of noninsecticidal strain 14577 and insecticidal strain 1593 were examined (Table 2). Whole-cell preparations of strain 14577 were able to utilize substrates Ile-β-NA and Val-β-NA while whole-cell preparations of strain 1593 were not. Cell-free preparations of strain 14577 were able to utilize substrates OH-Pro-β-NA, Ile-β-NA, Ser-β-NA, Val-β-NA, Gly-Gly-β-NA and Leu-Gly-β-NA, while cell-free preparations of strain 1593 did not. In terms of whole-cell vs. cell-free preparations, strain 14577 cell-free preparations were able to utilize substrates OH-Pro-β-NA and Ser-Tyr-β-NA, while whole-cell preparations of the same strain could not. Cell-free preparations of strain 1593 could utilize substrate Ser-Tyr-β-NA, while whole-cell preparations could not. Conversely, whole-cell preparations of 1593 could utilize substrates Ser-β-NA, Gly-Gly-β-NA and Leu-Gly-β-NA while cell-free preparations of strain 1593 could not. Sampling time had an effect on substrate utilization, whether examining whole-cell or cell-free preparations. Generally speaking, where substrate utilization was noted it occurred at the later time periods (12 and/or 20 h). The only exception to this was in the utilization of Ser-Tyr-β-NA by cell-free preparations of strain 14577, where utilization of the substrate occurred at 3 and 12 h but not 20 h.

Polyacrylamide gels

Four substrates were chosen from the aminopeptidase SAP-II and SAP-III classes of β-naphthyl-

Table 2

Comparison of the effect of sampling time on aminopeptidase (SAP-III) enzyme activity in whole cells and cell-free extracts of *B. sphaericus*

Substrate	Whole cells						Cell-free extracts					
	14577			1593			14577			1593		
	3[a]	12	20	3	12	20	3	12	20	3	12	20
His-β-NA	−	−	−	−	−	−	−	−	−	−	−	−
OH-Pro-β-NA	−	−	−	−	−	−	−	+	+	−	−	−
Ile-β-NA	−	−	+	−	−	−	−	+	+	−	−	−
Met-β-NA	+	+	+	+	+	+	−	+	+	−	−	+
Ser-β-NA	+	+	+	+	+	+	+	+	+	−	−	−
Tyr-β-NA	−	−	−	−	−	−	−	−	−	−	−	−
Trp-β-NA	−	−	−	−	−	−	−	−	−	−	−	−
Val-β-NA	−	+	+	−	−	−	−	+	+	−	−	−
Gly-Gly-β-NA	+	+	+	−	+	+	−	+	+	−	−	−
Gly-Phe-β-NA	+	+	+	+	+	+	+	+	+	+	+	+
Leu-Gly-β-NA	+	+	+	−	+	+	−	+	+	−	−	−
Ser-Tyr-β-NA	−	−	−	−	−	−	+	+	−	+	+	+

[a] Age of cells in h.

−, no substrate hydrolysis; +, substrate hydrolysis.

Table 3

Aminopeptidase electromorph R_f values from eight *B. sphaericus* strains

Strain	Substrate hydrolyzed											
	Ala-β-NA		Leu-β-NA				Ser-β-NA			Gly-Phe-β-NA		
	1[a]	2	1	2	3	4	1	2	3	1	2	3
14577	0.573	0.181	–	0.547	0.170	0.161	0.547	–	0.153	0.598	0.145	0.061
7054	0.589	0.183	–	0.563	0.179	0.170	0.569	–	0.162	0.573	0.173	0.055
1593	0.593	0.195	0.583	0.560	0.179	–	0.576	0.171	0.157	0.586	0.173	0.055
2297	0.593	0.199	–	0.578	0.196	–	0.583	–	0.170	0.586	0.173	0.055
2500	0.593	0.199	0.871	0.580	0.196	–	0.581	0.171	0.161	0.604	0.167	0.063
SSII-1	0.598	0.210	–	0.595	0.203	–	0.584	–	0.168	0.606	0.165	0.046
2362	0.595	0.198	–	0.595	0.203	–	0.583	–	0.162	0.580	0.166	0.051
2013-4	0.551	0.184	–	0.555	0.182	–	0.571	–	0.151	0.613	0.170	0.057
Electromorph average	0.585	0.192	0.862	0.577	0.189	0.166	0.575	0.171	0.161	0.598	0.167	0.052

[a] Locus.
–, no electromorph detected.

amide substrates for electrophoretic analysis of enzyme activity (Table 3). Of the eight strains examined, two to four loci were noted for the substrates: two loci for Ala-β-NA, four loci for Leu-β-NA and three each for Ser-β-NA and Gly-Phe-β-NA. With the exception of locus 2 of Ser-β-NA, which proved monomorphic, all of the loci were polymorphic, exhibiting different alleles among strains.

Statistical analysis

Calculation of percent similarities of the *B. sphaericus* strains resulted in the matrix shown in Table 4. These data were utilized in a cluster analysis [12] to produce the dendrogram in Fig.1.

DISCUSSION

Analysis of aminopeptidase activity in commercially available enzyme panels (SAP-II and SAP-III) indicated qualitative differences among the eight strains of *B. sphaericus* tested (Table 1). Dif-

Table 4

Percent similarity between eight *B. sphaericus* strains [12]

Strain	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
(1) 14577		78.2	55.1	46.6	55.4	52.2	57.0	66.2
(2) 7054			63.6	53.9	61.2	45.2	57.4	77.8
(3) 1593				63.0	72.2	66.0	81.0	80.8
(4) 2297					64.7	65.1	67.3	72.9
(5) 2500						67.3	80.0	79.2
(6) SSII-1							68.6	76.1
(7) 2362								88.0
(8) 2013-4								

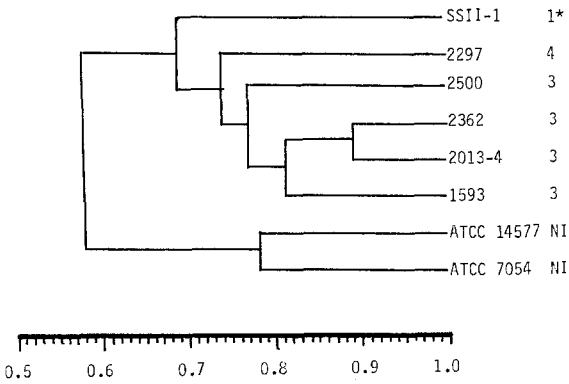

Fig. 1. Dendrogram of percent similarities between eight *B. sphaericus* strains determined by the UPGMA [20]. Horizontal axis is the percent similarity, vertical axis is the strains. * Phage type; NI = noninsecticidal.

ferentiating bacteria by the specificity of their aminopeptidase activities was first introduced by Westley et al. [23]. Since then, the technique has been applied to fungal pathogens of plants [8], as well as to other pathogens such as yeast, protozoa and intestinal worms [22], and to bacteria [2].

The qualitative differences in aminopeptidase activity seen in this study involved specific substrates utilized (Table 1), the importance of sampling time, as well as differences between whole cells and cell-free extracts (Table 2). It has been well documented that variation in the sampling time (during the growth cycle) affects the substrate specificity of aminopeptidase enzymes [7,21,22]. Therefore, for reproducibility of enzyme patterns for the identification and comparison purposes, sampling time must remain constant. In this study, there was an increase in substrate hydrolysis when the cultures were sampled later in the growth cycle.

There was an increase in enzyme activity when the whole cells were in stationary phase (12, 20 h) as compared to cells sampled at 3 h (log phase of growth) (Table 2). *B. sphaericus*/14577 hydrolyzed Ile-β-NA only at 20 h and Val-β-NA at 12 and 20 h, while *B. sphaericus*/1593 did not hydrolyze these two substrates at any sampling time. Also, strain 1593 hydrolyzed Gly-Gly-β-NA and Leu-Gly-β-NA at 12 and 20 h sampling times. When cell-free extracts of *B. sphaericus*/14577 were examined, there was activity at 12 and 20 h for Ile-β-NA, while activity in the whole cells was only observed at 20 h. This indicated that the enzyme was synthesized at or before 12 h but was not active until 20 h (Table 2).

The eight cultures examined in this study are of independent origin and have been cultivated under independent conditions. It is reasonable to expect that many base changes in the DNA of each strain may have accumulated; however, those which did not affect the conserved active centers of the enzymes might result in the evolutionary divergence of the proteins, and these might exhibit markedly different electrophoretic mobilities of R_f values [2].

Further detailed analysis of four of the L-amino acid-β-naphthylamide substrains using polyacrylamide gel electrophoresis demonstrated a series of allelic differences in the multilocus patterns observed (Table 3). Cluster analysis (Table 4) and the resulting dendrogram constructed from this information (Fig. 1) showed a marked separation of the noninsecticidal strains from the insecticidal strains (58% similarity) while the two noninsecticidal strains were 78% similar. In addition, there is a separation within the insecticidal strains of strain SSII-1, phage type 2 [25] from strain 2297, phage type 4 [25], and of both of these strains from the remaining highly insecticidal members of phage type 3 [24,25].

The zymogram technique, which consists of zone electrophoresis followed by staining in situ for specific enzyme activity [9], provides a sensitive method for the detection of minor structural differences among enzymes [16]. This study was a preliminary effort to differentiate phenotypically insecticidal and noninsecticidal strains of *B. sphaericus* based upon aminopeptidase mobility differences once it was established from preliminary studies, using enzyme panels, that differences occurred in aminopeptidase substrate utilization. An extension of the zymogram technique, using many other enzymes to differentiate between and within the insecticidal and noninsecticidal groups of strains of *B. sphaericus,* is presently in progress in our laboratory.

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