Antibiotic Susceptibility as a Taxonomic Characteristic of the Genus *Bacillus*

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A large number of *Bacillus* strains assigned to different species were tested to determine their susceptibilities to antibiotics. Some clear differences between species were observed. The antibiotic susceptibilities of strains isolated from natural sources seemed to be stable and to reflect adaptation of the strains to specific conditions in certain ecological niches. A method for data processing which can be used for rapid species identification is described.

Bacteria belonging to the genus *Bacillus* are among the most widely distributed microorganisms and play significant roles in microbial communities. Nevertheless, species identification of microorganisms belonging to the genus *Bacillus* is very difficult because of the extreme phenotypic variability of these organisms. The most complete characterization of *Bacillus* species has been published by Priest et al. (9, 10). It is notable that the number of phenotypic tests by which the species can clearly be distinguished from each other is very small.

The susceptibility of bacilli to different antibiotics has been studied previously, and it has been demonstrated that in principle it should be possible to identify species on the basis of the results of susceptibility tests (3, 4, 6, 7, 11). However, a simple method for data processing has not been available previously.

The instability of the plasmids that are present in *Bacillus* cells can cause doubts concerning the taxonomic value of susceptibility to antibiotics (1, 8, 10). Previously, it was thought that the resistance of natural isolates was a stable property because it reflected adaptation of strains to conditions in specific ecological niches occupied by certain species.

The purpose of this study was to develop a method for identification based on a statistical evaluation of species differences in antibiotic susceptibility.

In this study we used 110 strains belonging to 17 species of the genus *Bacillus*. Species were identified as described by Claus and Berkeley (5) by using the following characteristics: cellular and spore morphology; Voges-Proskauer test; nitrate reduction to nitrite; anaerobic growth; gas production from

nitrate and glucose; starch, urea, and casein degradation; acid production from glucose, galactose, lactose, mannose, and mannitol; utilization of citrate, acetate, lactate, and malonate; phenylalanine deamination; and arginine dihydrolase activity.

The test used to determine antibiotic susceptibility was performed by the disk agar diffusion method on Gause-2 medium containing (per liter of distilled water) 30 ml of Chottinger broth, 5 g of peptone, 5 g of NaCl, 10 g of glucose, and 15 g of agar.

The nutrient medium was poured into 100-mm petri dishes. Then cultures were grown overnight on the nutrient agar at 37°C and were used to prepare suspensions having an optical density of 5 U (about 500,000 cells per ml). A 0.1-ml portion of each suspension was plated onto agar, and disks containing antibiotics were placed onto the surface of the medium. The cultures were incubated overnight at 37°C. After incubation the diameters of the zones of growth inhibition were measured. Disks containing monomycin (30 µg per disk), oleandomycin (15 µg), oxacillin (10 µg), ristomycin (30 µg), gentamicin (10 µg), chloramphenicol (30 µg), ampicillin (10 µg), erythromycin (15 µg), polymyxin (300 U), kanamycin (30 µg), lincomycin (15 µg), carbenicillin (25 µg), methicillin (10 µg), neomycin (30 µg), tetracycline (30 µg), streptomycin (30 µg), and benzylpenicillin (10 U) were used.

On the basis of the results of our identification tests, 75 fresh *Bacillus* strains were placed in the following six species: *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus cereus*, *Bacillus megaterium*, and *Bacillus polymyxa*.

TABLE 1. Means and standard deviations of inhibition zone diameters^a

Species	No. of strains	Diam of inhibition zone (mm) with:							
		Oleandomycin	Oxacillin	Chloramphenicol	Ampicillin	Carbenicillin	Ristomycin	Tetracycline	Benzylpenicillin
<i>B. subtilis</i>	30	20 ± 1	27 ± 2	25 ± 2	21 ± 2	23 ± 2	18 ± 2	17 ± 3	19 ± 3
<i>B. licheniformis</i>	10	10 ± 4	15 ± 3	17 ± 3	18 ± 2	16 ± 3	17 ± 1	23 ± 6	16 ± 3
<i>B. pumilus</i>	10	20 ± 2	26 ± 5	20 ± 3	30 ± 5	29 ± 4	14 ± 2	30 ± 3	32 ± 7
<i>B. cereus</i>	10	17 ± 2	7 ± 1	21 ± 3	7 ± 1	7 ± 1	16 ± 1	17 ± 4	7 ± 1
<i>B. megaterium</i>	10	15 ± 3	22 ± 2	22 ± 3	21 ± 3	24 ± 2	16 ± 2	23 ± 3	23 ± 2
<i>B. polymyxa</i>	10	9 ± 3	20 ± 6	31 ± 7	27 ± 9	31 ± 10	21 ± 3	34 ± 10	28 ± 10

^a Standard deviations were calculated for $P = 0.99$.

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TABLE 2. Coefficients of linear discriminant functions

Function	Coefficient with:							
	Oleandomycin	Oxacillin	Chloramphenicol	Ampicillin	Carbenicillin	Ristomycin	Tetracycline	Benzylpenicillin
First	0.08	-0.05	0.02	0.43	0.17	-0.01	0.14	-0.02
Second	-0.28	-0.58	0.05	0.43	-0.27	0.85	0.40	0.12

A comparison of the diameters of the zones of growth inhibition revealed that the susceptibilities of all of the strains to the antibiotics monomycin, kanamycin, gentamicin, erythromycin, methicillin, neomycin, and streptomycin were nearly the same. Most of the strains studied were resistant to lincomycin. Low levels of susceptibility to polymyxin (diameters of zones of inhibition, 10 to 18 mm) were observed with strains related to *B. megaterium*; this characteristic distinguished these organisms from other species that were completely resistant to this antibiotic. Our data are consistent with previously published

data (3, 6-10). The specific variations in methicillin susceptibility and streptomycin susceptibility observed previously (6, 10) were not confirmed by our results. The levels of susceptibility to these antibiotics varied widely for different strains, as the diameters of the zones of inhibition for methicillin and streptomycin were 16 to 32 and 10 to 24 mm, respectively; however, there was no relationship between this characteristic and species identity.

Considerable interspecific differences in susceptibility were observed with oleandomycin, oxacillin, chloramphenicol, ampicillin, carbenicillin, ristomycin, tetracycline, and benzylpenicillin.

The means and standard deviations for inhibition zone diameters determined for 75 fresh isolates belonging to different *Bacillus* species are shown in Table 1. We found that the differences between species were greater than the differences within individual species. Thus, levels of susceptibility to certain groups of antibiotics may be considered suitable characteristics for *Bacillus* species identification. We found that the levels of interspecific variance calculated for each of eight antibiotics ranged from 70 to 90% of the whole-sample levels of variance; this was interpreted as being sufficient for adequate species identification.

TABLE 3. Inhibition zone diameters for collection strains

Species or subspecies	Strain	Diam of inhibition zone (mm) with:								Coordinates	
		Oleandomycin	Oxacillin	Chloramphenicol	Ampicillin	Carbenicillin	Ristomycin	Tetracycline	Benzylpenicillin	First axis	Second axis
<i>B. subtilis</i>	ATCC 10774	20	28	28	22	22	16	15	20	15.5	5.1
	CCEB 115	18	30	28	24	25	16	17	21	16.9	5.5
	VKM 501	21	27	27	27	28	18	11	20	18.2	6.0
	VKM 2895	22	30	27	25	25	17	19	21	17.9	6.4
	VKM 2896	22	30	28	22	25	17	16	24	16.1	4.3
<i>B. subtilis</i> subsp. <i>niger</i>	CCM 110	19	24	18	20	20	18	24	14	15.6	11.4
<i>B. licheniformis</i>	ATCC 14580	13	17	19	20	20	18	24	14	15.5	17.2
	CIP 5126	13	18	25	19	18	17	28	18	15.3	18.3
	CIP 5829	Res ^a	13	15	13	15	16	20	10	10.6	25.1
	IFO 12200	12	15	18	19	16	20	26	16	14.6	22.0
	VKM 511	15	22	19	19	16	20	26	20	14.4	17.6
<i>B. pumilus</i>	CCM 2614	19	20	15	20	28	15	31	30	17.8	13.6
	CCM 2218	20	20	15	26	28	15	29	28	20.2	15.9
	VKM 508	18	36	18	42	34	18	36	46	27.8	19.1
<i>B. polymyxa</i>	ATCC 842	14	22	19	24	30	14	25	26	18.7	11.5
	VKM 504	14	18	25	23	22	20	20	26	16.4	19.0
<i>B. alvei</i>	VKM 724	25	28	32	27	30	19	28	29	21.1	12.7
<i>B. badius</i>	VKM 496	25	32	26	40	42	19	22	38	27.6	10.8
<i>B. cereus</i>	IMV 23570	17	8	22	Res	Res	16	24	Res	8.1	16.6
	IMV 211	21	Res	23	Res	Res	15	9	Res	6.4	9.8
<i>B. circulans</i>	VKM 693	23	Res	22	13	21	18	24	12	14.1	17.4
<i>B. laterosporus</i>	VKM 499	21	30	26	30	31	18	25	27	21.7	11.1
<i>B. pasteurii</i>	VKM 1063	20	21	20	29	31	16	20	26	20.8	12.0
<i>B. pulvificiens</i>	VKM 748	15	18	24	18	18	19	25	15	14.6	17.4
<i>B. maceans</i>	NCIB 1068	21	15	27	22	30	22	16	32	17.4	17.1
<i>B. megaterium</i>	KSU 164	18	24	25	22	22	15	21	20	16.3	9.4
<i>B. mycoides</i>	IMV 1996	Res	19	20	18	15	20	13	17	14.4	27.0
<i>B. sphaericus</i>	ATCC 14577	18	23	20	25	23	15	39	25	20.2	18.5
	VKM 509	13	14	15	10	26	11	17	20	11.2	4.8
<i>B. stearothermophilus</i>	IMV 360	Res	17	15	20	14	17	27	10	11.9	23.8
<i>B. thuringiensis</i> subsp. <i>sotto</i>	VKM 453	23	Res	29	13	13	20	23	9	12.8	20.9
<i>B. thuringiensis</i>	VKM 740	17	25	25	32	34	13	19	34	22.0	9.3

^a Res, resistant.

FIG. 1. Locations of *Bacillus* species on a plane.

We observed a broad spectrum of values for the levels of correlation between antibiotics, and the correlation matrix formed the database for a principal-component analysis (2, 9). Two linear discriminant functions were calculated. The coefficients of these functions are shown in Table 2.

Values for the two linear discriminant functions are equal to the sums of the experimental values multiplied by the corresponding coefficients. For example, to calculate the levels of susceptibility of *B. subtilis* ATCC 10774 to antibiotics (Table 3), the first function is $(20 \times 0.08) - (28 \times 0.05) + (28 \times 0.02) + (22 \times 0.43) + (22 \times 0.17) - (16 \times 0.01) + (15 \times 0.14) - (20 \times 0.02) = 15.5$, and the second function is $(20 \times -0.28) - (28 \times 0.58) + (28 \times 0.05) + (22 \times 0.43) - (22 \times 0.27) + (16 \times 0.85) + (15 \times 0.4) + (20 \times 0.12) = 5.1$.

As a result, for each strain we obtained two values which can be considered coordinates of the strain on a plane. A graphic representation of the results of our calculations for all of the strains which we tested is shown in Fig. 1. Six *Bacillus* species were characterized by defined locations on the plane by using the coordinate limits shown in Table 4.

To evaluate the method which we used for identification, 32 strains that were assigned to different *Bacillus* species and were received from some national and private collections were tested by the procedure described above. The data obtained and the decisions reached based on the two discriminant functions are shown in Table 3. The values for *B. subtilis* ATCC 10774, CCEB 115, VKM 2895, and VKM 2896 were within the ranges obtained for *B. subtilis* strains belonging to group I (Fig. 1), and the values for *B. subtilis* subsp. *niger* CCM 110 were within the ranges obtained for the values for *B. subtilis* group II. Other strains belonging to group II were characterized by slimy growth and dark pigmentation, which distinguished them

from strains belonging to *B. subtilis* group I. These strains are probably related to *B. subtilis* subsp. *niger*.

The locations of the *B. licheniformis*, *B. pumilus*, *B. cereus*, and *B. megaterium* strains generally corresponded to the locations shown in Fig. 1, but the locations of *B. polymyxa* ATCC 842 and VKM 504 did not. It is possible that long-term storage of strains ATCC 842 and VKM 504 under laboratory conditions caused substantial changes in them.

Bacillus alvei VKM 724, *Bacillus badius* VKM 496, *Bacillus laterosporus* VKM 499, *Bacillus pasteurii* VKM 1063, *Bacillus macerans* NCIB 1068, *Bacillus mycoides* IMV 1996, *Bacillus sphaericus* ATCC 14577 and VKM 509, and *Bacillus thuringiensis* VKM 740 could be distinguished clearly from other species by their coordinates on the plane. In contrast, *Bacillus circulans* VKM 693, *Bacillus pulvifaciens* VKM 748, *Bacillus stearothermophilus* IMV 360, and *Bacillus thuringiensis* subsp. *sotto* VKM 453 merged with other species.

The method which we describe above is not considered an alternative to other biochemical and physiological tests used for conventional identification of bacilli. However, some significant additional information about taxonomic relationships among *Bacillus* species may be obtained from analyzing antibiotic susceptibilities.

The data available contain sufficient discriminatory power to allow quick species determinations or at least rough determinations. More precise identifications may be made by tests specific for each species; this should mean that workers can avoid the use of large numbers of biochemical tests for conventional identification.

Furthermore, some closely related species and morphological groups (for instance, *B. subtilis* and *B. subtilis* subsp. *niger*) can be differentiated.

Susceptibilities to certain antibiotics evidently reflect other forms of variation in bacilli and adaptation to certain environmental conditions. These relationships are important for understanding the place occupied by these species in microbial associations.

REFERENCES

- Bernhard, K., H. Schrempf, and W. Goebel. 1978. Bacteriocin and antibiotic resistance plasmids in *Bacillus cereus* and *Bacillus subtilis*. J. Bacteriol. 133:897-903.
- Brondz, I., I. Olsen, and M. Sjöström. 1990. Multivariate analysis of quantitative chemical and enzymic characterization data in classification of *Actinobacillus*, *Haemophilus* and *Pasteurella* spp. J. Gen. Microbiol. 136:507-513.
- Burke, W. F. J., and K. O. McDonald. 1983. Naturally occurring antibiotic resistance in *Bacillus sphaericus* and *Bacillus licheniformis*. Curr. Microbiol. 9:69-72.
- Castro, G. R., M. A. Ferrero, B. S. Mendez, and F. Sineriz. 1991. A system for the differentiation of some closely related *Bacillus* species. J. Biotechnol. 20:105-108.
- Claus, D., and C. W. Berkeley. 1986. Genus *Bacillus* Cohn 1872, p. 1105-1139. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.
- Coonrod, J. D., P. J. Leadley, and T. C. Eickhoff. 1971. Antibiotic susceptibility of *Bacillus* species. J. Infect. Dis. 123:102-105.
- Denis, F., M. Geslin, and A. Nivet. 1975. Sensibilité aux antibiotiques des différents espèces de *Bacillus* (étude de 400 souches). Quest. Sci. Med. 22:1629-1633.
- Jimenez-Valera, M., A. Puiz-Bravo, and A. Ramos-Cormezana. 1985. Posible interés taxonomico de las pruebas de sensibilidad frente a antibióticos en el género *Bacillus*. Ars Pharm. Rev. Fac. Farm. 26:157-163.
- Priest, F. G., and B. A. Alexander. 1988. A frequency matrix for the probabilistic identification of some bacilli. J. Gen. Microbiol. 134:3011-3018.
- Priest, F. G., M. Goodfellow, and C. Todd. 1988. Numerical classification of the genus *Bacillus*. J. Gen. Microbiol. 134:1847-1882.
- Rosswall, T., and E. Kvillner. 1978. Principal-component and factor analysis for the description of microbial populations, p. 1-48. In M. Alexander (ed.), Advances in microbial ecology. Plenum Press, New York.
- Scavizzi, M., L. Stuart, and A. Gordon. 1991. Un modèle statistique d'interprétation de l'antibiogramme. C. R. Acad. Sci. Ser. 3 304:325-329.

TABLE 4. Coordinate limits for *Bacillus* species on a plane

Taxon	No. of strains	Coordinate limits	
		First axis	Second axis
<i>B. subtilis</i> group I	21	14.0-20.0	1.0-8.0
<i>B. subtilis</i> group II	7	14.0-20.0	10.0-13.0
<i>B. licheniformis</i>	10	10.0-16.0	15.0-25.0
<i>B. pumilus</i>	10	19.0-28.0	13.0-19.0
<i>B. cereus</i>	10	3.0-10.0	10.0-17.0
<i>B. megaterium</i>	10	14.0-19.0	10.0-13.0
<i>B. polymyxa</i>	5	15.0-25.0	24.0-33.0