Characterization of *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* by Pyrolysis Gas-Liquid Chromatography, Deoxyribonucleic Acid-Deoxyribonucleic Acid Hybridization, Biochemical Tests, and API Systems

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Eight strains each of *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* were analyzed by using pyrolysis gas-liquid chromatography. Statistical analysis with canonical variates gave four well-separated groups, which represented the four species. Further analysis of the same strains by deoxyribonucleic acid-deoxyribonucleic acid hybridization and API identification systems confirmed the discrimination obtained with pyrolysis gas-liquid chromatography. However, analysis by biochemical tests performed in the classical way gave only three groups since it was not possible to achieve separation of the strains representing *B. subtilis* from those of *B. amyloliquefaciens* when these tests were used.

Pyrolysis, a process whereby molecules are thermally degraded in an inert gas atmosphere, has enhanced the use of conventional gas-liquid chromatography by enabling nonvolatile compounds to be analyzed. Pyrolysis gas-liquid chromatography (PGLC) was first proposed as an approach to microbial differentiation by Oyama (15) during the development of a system aimed at detecting life on Mars. However, its potential in microbiology was not appreciated until Reiner (17) was able to distinguish different species of *Mycobacterium* and different serotypes of *Escherichia coli* in a reproducible manner. Since then, PGLC has been used in the differentiation of numerous types of bacteria (10, 18, 22) and fungi (5, 23). The recent application of PGLC to aerobic sporeformers by Oxborrow et al. (12-14) indicates that, providing the cultural and chromatographic conditions remain constant, PGLC can be applied usefully to the characterization of bacilli.

The variation between pyrograms of the same strain and the high level of redundancy found in PGLC data require the application of data processing techniques capable of highlighting significant variations in the heights of specific peaks. Several methods for handling data in this

way have been described (10, 16), but as yet there is no agreement on the best statistical approach, and much work remains to be done in this field.

This paper reports on the usefulness of low-resolution PGLC when coupled to multivariate data analysis for differentiating closely related groups of bacteria and provides evidence for the separation of *Bacillus amyloliquefaciens* from *Bacillus subtilis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The majority of the strains used (Table 1) were from the collection of the late T. Gibson; the cultures were held on soil extract agar slants at the Meat Research Institute. Also included were strains from the American Type Culture Collection, the Deutsche Sammlung von Mikroorganismen, and T. Kaneko. Organisms were grown on membrane filters (type HAWP 047; 0.45 µm; Millipore Corp.), as described by Oxborrow et al. (12). Each culture was incubated for 14 h at 30°C on nutrient agar (Oxoid) containing 2 g of glucose per liter. Cultures showing sporulation were replated. Only non-sporulated cultures were used for PGLC analysis.

Examination of strains by PGLC. (i) Sample preparation. Samples were harvested from the membrane filters by using a sterile platinum loop and were stored in sterile distilled water at -4°C before analysis. Bacterial suspensions containing 80 to 100 µg of cells were applied directly to the platinum coil of a Chem-

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TABLE 1. *Bacillus* strains used in this study

Strain no. in study	MRI no. ^a	Identity	Comments ^b
1	32	<i>B. subtilis</i>	DSM 10 (neotype)
2	38	<i>B. subtilis</i>	Gibson 636
3	39	<i>B. subtilis</i>	Gibson 1111
4	40	<i>B. subtilis</i>	Gibson 1156
5	41	<i>B. subtilis</i>	Gibson 1137
6	42	<i>B. subtilis</i>	Gibson 1115
7	43	<i>B. subtilis</i>	Gibson 1136
8	44	<i>B. subtilis</i>	Gibson 1152
9	37	<i>B. pumilus</i>	DSM 27 (type)
10	58	<i>B. pumilus</i>	Gibson 1130
11	59	<i>B. pumilus</i>	Gibson 1036
12	60	<i>B. pumilus</i>	Gibson 10
13	61	<i>B. pumilus</i>	Gibson 47
14	62	<i>B. pumilus</i>	Gibson 67
15	63	<i>B. pumilus</i>	Gibson 604
16	64	<i>B. pumilus</i>	Gibson 768
17	35	<i>B. licheniformis</i>	DSM 13 (neotype)
18	49	<i>B. licheniformis</i>	Gibson 307
19	50	<i>B. licheniformis</i>	Gibson 1142
20	51	<i>B. licheniformis</i>	Gibson 1174
21	52	<i>B. licheniformis</i>	Gibson 46
22	53	<i>B. licheniformis</i>	Gibson 1160
23	54	<i>B. licheniformis</i>	Gibson 5
24	55	<i>B. licheniformis</i>	Gibson 1158
25	72	<i>B. amyloliquefaciens</i>	From Kaneko as <i>B. megaterium</i> 203
26	73	<i>B. amyloliquefaciens</i>	From Kaneko as Fukumoto strain F
27	74	<i>B. amyloliquefaciens</i>	From Kaneko as <i>B. subtilis</i> H
28	75	<i>B. amyloliquefaciens</i>	From Kaneko as <i>B. subtilis</i> K
29	76	<i>B. amyloliquefaciens</i>	From Kaneko as <i>B. subtilis</i> N
30	95	<i>B. amyloliquefaciens</i>	From Gordon as ATCC 23843
31	96	<i>B. amyloliquefaciens</i>	From Gordon as ATCC 23845
32	97	<i>B. amyloliquefaciens</i>	From Gordon as ATCC 23842

^a MRI, Meat Research Institute.^b DSM, Deutsche Sammlung von Mikroorganismen; ATCC, American Type Culture Collection.

ical Data Systems 190 pyroprobe by using a microsyrringe (Hamilton). Repeated firing of the probe in air at 50°C ensured evaporation of excess water.

(ii) **Chromatography.** Chromatographic analysis was carried out with a Perkin-Elmer F17 gas chromatograph fitted with dual glass columns (3 m by 5-mm inside diameter) packed with 10% Carbowax 20M-TPA on Chromosorb W 85-100 mesh AW-DMCS (Phase Separations Ltd., Queensferry, England). Pyrolysis was carried out in a stream of nitrogen (20 ml/min) at 850°C for 10 s. An injection temperature of 250°C was used. Refiring of a clean probe resulted in no shadow chromatograms. After an initial hold at 75°C for 2 min, the oven temperature was increased 10°C/min to 200°C and held at that temperature. Raising the temperature to 230°C after an analysis removed the compounds with higher boiling points, thereby cleaning the column. The total analysis time was approximately 50 min. Output from the column was detected by a flame ionization detector with an attenuation of 32 and was recorded at 1 cm/min on two parallel chart recorders set at full-scale deflections of 2 and 5 mV.

(iii) **Data collection.** Each culture was plated in duplicate, and the suspensions from each plate were analyzed twice. A base line was set manually across

each cluster of peaks in each pyrogram. Although setting the base line was an arbitrary procedure, once established for this study, it was set for all of the pyrograms in the same way (Fig. 1). A set of 23 reproducibly resolved peaks was chosen, and their heights were measured to the nearest millimeter. The criteria for choosing these peaks were that they showed the same relative retention time on each pyrogram and that their heights could be measured in every case. To remove variation due to sample size, these 23 peaks were standardized to a common total peak height. This was done by dividing each of the 23 peaks on each pyrogram by the sum of the 23 peaks and multiplying the quotient by 1,000. In this way pyrograms of different sample sizes could be compared. The standardized data were analyzed by using the ICL system 4/70 computer at Rothamsted Experimental Station. Figures 2 and 3 show the mean peak heights of all of the pyrograms for each species (i.e., the species means) and illustrate the qualitative similarity and high redundancy of standardized peak height data from PGLC. Since it was not possible to select a single peak that differentiated all of the species, it was necessary to use statistical methods which used several or all of the peaks simultaneously.

Data analysis: canonical variates. Each pyro-

FIG. 1. Pyrogram of *B. amyloliquefaciens* strain 26 showing base line and chosen set of peaks.

FIG. 2. Line diagrams representing the mean peak heights for *B. subtilis* and *B. amyloliquefaciens*. These means were derived from all of the analyses used to define each group.

gram with its particular set of peak heights (in this case 23) can be represented as a single point in multidimensional space, with each peak height representing one dimension of that space. In this study the 32 sets of coordinates representing the strain means define a multidimensional scatter; only two or three dimensions of this scatter can be represented visually. Canonical variates analysis redefines the distances between groups of points in terms of Mahalanobis D^2 (a concept taking into account the scatter of samples around the mean) and plots the directions of maximum variation. In this way the best two-dimensional picture of a multidimensional configuration is obtained (Fig. 4). The application of this program has been discussed previously by MacFie et al. (10), and a more detailed

description is given by Marriott (11) and Blackith and Reymont (2).

DNA-DNA hybridization. For deoxyribonucleic acid (DNA)-DNA hybridizations, organisms were grown in a medium consisting of 5 g of polypeptone (Wako Pure Chemical Industries Co.) per liter, 5 g of beef extract (Wako Pure Chemical Industries Co.) per liter, and 2 g of yeast extract (Difco Laboratories) per liter. For labeling of DNA, [*methyl*- ^3H]thymidine (0.5 mCi in 100 ml) was added. Of the strains used as references (Table 2), *B. subtilis* 168 required thymine, which was added at 5.0 and 1.5 mg/liter for the preparations of unlabeled and labeled DNAs, respectively. An overnight culture was added to 10 times its volume of fresh medium and was shaken at 37°C for 2.5 to 4

FIG. 3. Line diagrams representing the mean peak heights for *B. pumilus* and *B. licheniformis*. These means were derived from all of the analyses used to define each group.

FIG. 4. Plot of the strain means of the 32 organisms used relative to the first two canonical variate axes. Strains 1 through 8, *B. subtilis*; strains 9 through 16, *B. pumilus*; strains 17 through 24 *B. licheniformis*; strains 25 through 32, *B. amyloliquefaciens*. Coincident strains are marked with a superscript plus sign.

h before washing with 0.1 M sodium ethylenediamine-tetraacetate (pH 8.0). After washing, the cells were stored at -20°C .

The DNA was extracted as described by Saito and Miura (19) and was treated twice with ribonuclease A. In the hybridization experiments, a membrane filter bearing 50 μg of unlabeled, denatured DNA was incubated at 65°C for 64 h in 1 ml of a solution containing 0.3 M sodium chloride, 30 mM trisodium citrate, 0.1% dodecyl sulfate, and 4×10^6 to 6×10^8 cpm of labeled, heat-denatured DNA. Experiments were performed in triplicate, and hybrids were quantified by determining radioactivity of the filter paper after washing with 5

mM tris(hydroxymethyl)aminomethane (pH 9.5).

For certain organisms, particularly members of the *B. subtilis* and *B. amyloliquefaciens* groups, these conditions did not provide data which allowed an unambiguous characterization of species. When this occurred, an additional experiment, which tested the stability of the hybrids, was carried out. Filters used in the above-mentioned experiment were taken out of the scintillation mixture, soaked for 2 h in toluol, and air dried. They were then heated at 75°C for 1 h in a solution containing 0.15 M sodium chloride, 15 mM disodium citrate, and 0.1% sodium dodecyl sulfate and were washed and counted again.

TABLE 2. DNA-DNA hybridization reference strains

Strain	Comments
<i>B. amyloliquefaciens</i> F	Type strain.
<i>B. brevis</i> ATCC 8246	Neotype strain; guanine plus cytosine content similar to that of test organisms.
<i>B. licheniformis</i> ATCC 14580	Neotype strain.
<i>B. pumilus</i> ATCC 7061	Type strain.
<i>B. subtilis</i> 168	A thymineless derivative of the Marburg strain (H. Saito and F. Rothman) ^a

^a See reference 7.

Biochemical tests. Organisms were examined by a number of tests, as described by Gordon et al. (8) for the identification of *Bacillus* species, with the modifications listed below. Stock cultures were kept on nutrient agar containing 10 mg of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ per 1,000 ml, and each medium was inoculated by a loopful of culture grown at 30°C in nutrient broth for 45 h. Unless stated otherwise, incubation was at 30°C.

(i) **Catalase production.** Cultures grown on nutrient agar slants for 1 or 2 days were flooded with 3 ml of 10% hydrogen peroxide and were observed for gas production.

(ii) **Anaerobic growth.** A tube of nutrient broth supplemented with 1% (wt/vol) glucose was incubated in a GasPak anaerobic system (BBL Microbiology Systems). Growth (turbidity) was observed after 7 and 14 days. An aerobic culture served as a control for the suitability of inoculum and medium.

(iii) **Egg yolk reaction.** Egg yolk agar plates were prepared by mixing 50 ml of egg yolk emulsion (Oxoid) with sterilized nutrient agar containing 1% sodium chloride at 45°C and immediately pouring the mixture into petri dishes. An opaque zone around the colonies after 4 days of incubation at 30°C was considered to be a positive test.

(iv) **Maximum temperature for growth.** Instead of soil extract agar, nutrient agar was used.

(v) **Hydrolysis of starch.** Plates were developed with Gram iodine instead of ethanol.

(vi) **Citrate utilization.** The medium used consisted of the following: trisodium citrate (2 hydrate) ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$), 1 g; potassium chloride, 1 g; $\text{MgSO}_4 \cdot 2\text{H}_2\text{O}$, 1.2 g; diammonium hydrogen phosphate, 0.5 g; agar, 15 g; 0.04% (wt/vol) solution of phenol red, 20 ml; and distilled water, 1,000 ml.

API systems. The API 20E and 50E systems (API Laboratory Products Ltd., Farnborough, England) are standardized, miniaturized versions of conventional tests for the identification of *Enterobacteriaceae* and other gram-negative bacteria. They are ready-to-use microtube systems developed from the Buisson (3) modification of the Ivan Hall tube and contain 69 standard biochemical tests (Table 3). Eight tests are common to the two systems.

The API ZYM systems are semiquantitative micro-methods designed for the detection of enzymatic activities in a wide variety of specimens. They are a development of the Auxotab system described by Buisson et al. (4). The API ZYM strip contains 19 test substrates. An additional four strips (ZYM II, ZYM AP [aminopeptidase] 1, AP2, and AP3), which each contain 10 test substrates (9 substrates and 1 control in

the case of AP2), are not commercially available. The 58 enzyme tests include 32 aminopeptidases, 16 glycosidases, 3 phosphatases, 4 esterases, 2 proteinases, and 1 phosphoamidase.

Bacterial strains were grown on nutrient agar (Difco) plates incubated overnight at 30°C. Harvested cells were suspended in the following: (i) 10 ml of API ammonium salts medium, which contained 2 g of ammonium sulfate, 0.5 g of yeast extract, 10 ml of the mineral base of Cohen-Bazire et al. (6), and distilled water to 1 liter (pH 7) and corresponded to tube no. 2 of the MacFarland scale of standard opacities; (ii) 4 ml of normal saline, which corresponded to tube no. 2 of the MacFarland scale; and (iii) 6 ml of normal saline, which corresponded to tube no. 6 of the MacFarland scale.

The API 50E galleries and 20E strips were placed in plastic incubation chambers (previously moistened to maintain a humid atmosphere) and inoculated with suspensions i and ii, respectively. The last eight tests of the 20E strip were not inoculated because they were duplicated in the 50E gallery. The galleries and strips were incubated for 48 h at 30°C. Reactions were read at 24 and 48 h, and reagents were added, when necessary, at the second reading.

Enzyme test strips were placed in incubation chambers (as for 50E and 20E [see above]), and each cupule was inoculated with 0.05 ml of suspension iii. The strips were incubated in darkness at 30°C for 5 h. Under low-light conditions, 1 drop (0.025 ml) of 1 N NaOH was added to all but the first test of the ZYM II strip. Tests 2 to 8 were observed for a color reaction, and tests 9 and 10 were examined for fluorescence under ultraviolet light; 1 drop of API ZYM reagent A (buffer) and 1 drop of API ZYM reagent B (fast blue BB) were added to each cupule of the remaining strips. After 5 min any nonspecific yellowing of the color reagent was destroyed by exposure to bright daylight, and the color reactions were read with reference to the API ZYM color chart.

Data analysis. The results of the API tests were regarded as two-state characters. The general similarity coefficient of Gower (9) was used to compute similarities, and clustering was achieved by complete linkage by using the GENSTAT package.

RESULTS

The results of the canonical variates analysis performed on the pyrograms are shown in Fig. 4. This plot represents in two dimensions the scatter of the strain means in 23-dimensional

TABLE 3. *Tests of the API system used in this study*

System	Test no.	Test
API 20E	1	<i>o</i> -Nitrophenyl- β -D-galactopyranoside
	2	Arginine dihydrolase
	3	Lysine decarboxylase
	4	Ornithine decarboxylase
	5	Simmons citrate
	6	Hydrogen sulfide
	7	Urease
	8	Tryptophan deaminase
	9	Indole
	10	Voges-Proskauer
	11	Gelatin liquefaction
	12	Nitrate reduction
API 50E	0	Control
	1	Glycerol
	2	Erythritol
	3	D(-)-Arabinose
	4	L(+)-Arabinose
	5	Ribose
	6	D(+)-Xylose
	7	L(-)-Xylose
	8	Adonitol
	9	Methyl xyloside
	10	Galactose
	11	D(+)-Glucose
	12	D(-)-Fructose
	13	D(+)-Mannose
	14	L(-)-Sorbitol
	15	Rhamnose
	16	Dulcitol
	17	<i>meso</i> -Inositol
	18	Mannitol
	19	Sorbitol
	20	Methyl-D-mannoside
	21	Methyl-D-glucoside
	22	<i>N</i> -acetyl-glucosamine
	23	Amygdalin
	24	Arbutin
	25	Esculin
	26	Salicin
	27	D(+)-Cellobiose
	28	Maltose
	29	Lactose
	30	D(+)-Melibiose
	31	Saccharose
	32	D(-)-Trehalose
	33	Inulin
	34	D(+)-Melezitose
	35	D(+)-Raffinose
	36	Dextrin
	37	Amylose
	38	Starch
	39	Glycogen
	40	Methyl red
	41	Deoxyribonuclease
	42	Mucate
	43	Gluconate
	44	Lipase
	45	Tetrathionate reductase
	46	Pectate
	47	Citrate (Christensen)
	48	Malonate
	49	Acetate

TABLE 3—continued

System	Test no.	Test
API ZYM (enzyme test (substrates))	1	Control
	2	2-Naphthyl phosphate (pH 8.5)
	3	2-Naphthyl butyrate
	4	2-Naphthyl caprylate
	5	2-Naphthyl myristate
	6	L-Leucyl- β -naphthylamide
	7	L-Valyl- β -naphthylamide
	8	L-Cystyl- β -naphthylamide
	9	N-benzoyl-DL-arginine- β -naphthylamide
	10	N-benzoyl-DL-phenylalanine- β -naphthylamide
	11	2-Naphthyl phosphate (pH 5.0)
	12	Naphthol-AS-BI-phosphodiamide
	13	6-Br-2-naphthyl- α -D-galactopyranoside
	14	2-Naphthyl- β -D-galactopyranoside
	15	Naphthol-AS-BI- β -D-glucuronate
	16	2-Naphthyl- α -D-glucopyranoside
	17	6-Br-2-naphthyl- β -D-glucopyranoside
	18	1-Naphthyl-N-acetyl- β -D-glucosamide
	19	6-Br-2-naphthyl- α -D-mannopyranoside
	20	2-Naphthyl- α -L-fucopyranoside
ZYM II ^a	1	6-Br-2-naphthyl- β -D-xylopyranoside
	2	bis-p-Nitrophenyl phosphate
	3	p-Nitrophenyl- α -D-xylopyranoside
	4	p-Nitrophenyl- β -D-fucopyranoside
	5	p-Nitrophenyl- β -L-fucopyranoside
	6	o-Nitrophenyl-N-acetyl- α -D-glucosaminide
	7	p-Nitrophenyl lactoside
	8	p-Nitrocatechol sulfate
	9	4-Methylumbelliferyl-arabinopyranoside
	10	4-Methylumbelliferylcellobiopyranoside
API ^a	1	L-Tyrosyl- β -naphthylamide
	2	L-Pyrrolidonyl- β -naphthylamide
	3	L-Phenylalanine- β -naphthylamide
	4	L-Lysine- β -naphthylamide
	5	L-Hydroxyproline- β -naphthylamide
	6	L-Histidine- β -naphthylamide
	7	L-Glycine- β -naphthylamide
	8	L-Aspartyl- β -naphthylamide
	9	L-Arginyl- β -naphthylamide
	10	L-Alanyl- β -naphthylamide
AP2 ^a	1	α -L-Glutamyl- β -naphthylamide
	2	N-benzoyl-L-leucyl- β -naphthylamide
	3	S-benzyl-L-cysteine- β -naphthylamide
	4	DL-Methionyl- β -naphthylamide
	5	Glycyl-glycine- β -naphthylamide-HBr
	6	Glycyl-L-phenylalanyl- β -naphthylamide
	7	Glycyl-L-prolyl- β -naphthylamide
	8	L-Leucyl-L-glycyl- β -naphthylamide
	9	L-Seryl-L-tyrosyl- β -naphthylamide
	10	Control
AP3 ^a	1	NCBZ-L-arginine-3-methoxyl- β -naphthylamide
	2	L-Glutamine- β -naphthylamide
	3	α -L-Glutamyl- β -naphthylamide
	4	L-Isoleucine- β -naphthylamide
	5	L-Ornithine- β -naphthylamide
	6	L-Proline- β -naphthylamide
	7	L-Serine- β -naphthylamide
	8	L-Threonine- β -naphthylamide
	9	L-Tryptophan- β -naphthylamide
	10	NCBZ-Glycyl-glycyl-L-arginine- β -naphthylamide

^a Not commercially available at present.

TABLE 4. Strain identities as determined from DNA-DNA hybridization

Cold DNA from:	% Hybridization with Labeled DNA from:				Identity
	<i>B. amylo- liquefaciens</i>	<i>B. licheni- formis</i>	<i>B. pumilus</i>	<i>B. subtilis</i>	
Reference strains					
<i>B. amyloliquefaciens</i>	100	32	21	59	
<i>B. licheniformis</i>	34	100	21	32	
<i>B. pumilus</i>	17	17	100	22	
<i>B. subtilis</i>	50	38	29	100	
<i>B. brevis</i>	5	5	8	6	
Test strains					
1	37	17	15	94	<i>B. subtilis</i>
2	48	29	24	79	<i>B. subtilis</i>
3 ^a	91	33	24	61	<i>B. amyloliquefaciens</i>
4	45	30	22	107	<i>B. subtilis</i>
5	39	28	22	105	<i>B. subtilis</i>
6	51	36	26	90	<i>B. subtilis</i>
7	52	41	28	103	<i>B. subtilis</i>
8	47	44	29	98	<i>B. subtilis</i>
9	19	16	109	16	<i>B. pumilus</i>
10	24	15	96	24	<i>B. pumilus</i>
11	24	24	94	33	<i>B. pumilus</i>
12	29	19	85	32	<i>B. pumilus</i>
13	24	18	89	33	<i>B. pumilus</i>
14	24	20	76	30	<i>B. pumilus</i>
15	20	15	76	28	<i>B. pumilus</i>
16	29	18	77	24	<i>B. pumilus</i>
17	29	99	18	28	<i>B. licheniformis</i>
18	25	86	15	32	<i>B. licheniformis</i>
19	36	93	23	42	<i>B. licheniformis</i>
20	34	80	20	40	<i>B. licheniformis</i>
21	36	91	22	46	<i>B. licheniformis</i>
22	39	92	32	52	<i>B. licheniformis</i>
23	39	82	24	27	<i>B. licheniformis</i>
24	41	85	26	49	<i>B. licheniformis</i>
25	82	34	22	53	<i>B. amyloliquefaciens</i>
26	82	29	23	54	<i>B. amyloliquefaciens</i>
27	92	37	23	68	<i>B. amyloliquefaciens</i>
28	100	33	29	66	<i>B. amyloliquefaciens</i>
29	90	40	26	70	<i>B. amyloliquefaciens</i>
30	97	43	27	74	<i>B. amyloliquefaciens</i>
31	95	43	32	81	<i>B. amyloliquefaciens</i>
32	87	38	26	64	<i>B. amyloliquefaciens</i>

^a Strain labeled *B. subtilis* showed greater homology to *B. amyloliquefaciens*.

TABLE 5. Heat stability of hybrids

Unlabeled DNA from:	Stability ^a			
	Labeled DNA from <i>B. amyloliquefaciens</i>		Labeled DNA from <i>B. subtilis</i>	
	Not heated	Heated at 75°C for 1 h	Not heated	Heated at 75°C for 1 h
<i>B. amyloliquefaciens</i> reference	100	73	61	19
<i>B. licheniformis</i> reference			34	9
<i>B. subtilis</i> reference	43	13	100	77
Test strain 8	43	13	110	80
Test strain 31	97	70	72	22
Test strain 23	42	10	45	12
Test strain 30	96	67	64	21
Test strain 29	90	72	74	21

^a Figures indicate the degree of hybridization, expressed as a percentage of filter-bound reference DNA, after heating at 75°C.

FIG. 5. Cluster analysis of the API data. Strains 1 through 8, *B. subtilis*; strains 9 through 16, *B. pumilus*; strains 17 through 24, *B. licheniformis*; strains 25 through 32, *B. amyloliquefaciens*.

space and displays 98% of the total generalized variation among groups. Of particular interest is the separation of *B. subtilis* from *B. amyloliquefaciens*, which suggests that pyrolysis products of these groups are consistently different, thereby supporting the recognition of *B. amyloliquefaciens* as a species distinct from *B. subtilis*.

Table 4 shows the strain identities as determined on the basis of DNA-DNA hybridization. The relatively high homologies among *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus*, and *B. licheniformis* (compare values with those of *B. brevis*) support the generally accepted concept that these four species constitute a closely related group of organisms. In addition, these data show that *B. subtilis* and *B. amyloliquefaciens* are more related to one another than to *B. pumilus* or *B. licheniformis*. The experimental conditions employed in this investigation were not stringent enough, and as a result several strains appeared to be intermediates. This was due primarily to variation in the quality (filter-bound DNA not in duplex formation) and/or the quantity of the membrane-bound DNA. To overcome this, an additional experiment (see above), which tested the stability of the hybrids, was carried out. Table 5 shows some typical results. This experiment enabled a positive identification of all of the strains. No intermediates were found. In every case but one (strain 3), the

initial identity was confirmed, and the homology data suggested that *B. subtilis* and *B. amyloliquefaciens* are separate groups.

Figure 5 shows the results of applying cluster analysis to a series of API tests. The separation into groups agrees with the grouping obtained with PGLC and DNA-DNA hybridization and supports the separation of *B. subtilis* from *B. amyloliquefaciens*. However, two strains of *B. subtilis* (strains 2 and 3) form a small, separate cluster which joins the main cluster at a similarity of 65%. The tests (expressed as fractions positive) which we believe are responsible for this separation are shown in Table 6.

On the basis of the methods of Gordon et al. (8) (Table 7), it was possible to separate *B. pumilus* and *B. licheniformis* from each other and from *B. subtilis* and *B. amyloliquefaciens*. However, it was impossible to separate *B. subtilis* from *B. amyloliquefaciens* further.

DISCUSSION

B. subtilis, *B. pumilus*, and *B. licheniformis* represent a group of phenotypically related species known as the *B. subtilis* spectrum. When subjected to a battery of tests, strains representing these species share many common properties and show relatively few characteristics by which they can be separated (8).

Welker and Campbell described *B. amyloliquefaciens* as a species distinct from *B. subtilis*

TABLE 6. API system tests of value in differentiating *B. subtilis*, *B. pumilus*, *B. licheniformis*, and *B. amyloliquefaciens*

System	Strip no.	Test	No. positive/no. tested			
			<i>B. amyloliquefaciens</i>	<i>B. licheniformis</i>	<i>B. pumilus</i>	<i>B. subtilis</i>
20E	2	Arginine dihydrolase	0/8	7/8	0/8	0/8
	12	Nitrate reduction	6/8	8/8	0/8	8/8
50E	4	L(+)-Arabinose	2/8	8/8	8/8	6/8
	6	D(+)-Xylose	2/8	8/8	6/8	5/8
	10	Galactose	0/8	8/8	8/8	1/8
	15	Rhamnose	0/8	4/8	0/8	0/8
	17	meso-Inositol	4/8	6/8	1/8	8/8
	19	Sorbitol	8/8	8/8	1/8	7/8
	20	Methyl-D-mannoside	0/8	0/8	5/8	0/8
	21	Methyl-D-glucoside	8/8	8/8	3/8	8/8
	22	N-acetyl-glucosamine	6/8	8/8	8/8	2/8
	30	D(+)-Melibiose	2/8	0/8	3/8	5/8
	35	D(+)-Raffinose	8/8	2/8	4/8	6/8
	36	Dextrin	0/8	8/8	0/8	3/8
	38	Starch	8/8	7/8	0/8	8/8
	39	Glycogen	6/8	8/8	0/8	8/8
	43	Gluconate	7/8	8/8	8/8	2/8
API ZYM	10	Chymotrypsin	7/8	6/8	8/8	0/8
	13	α -D-Galactosidase	0/8	8/8	1/8	4/8
	14	β -D-Galactosidase	0/8	8/8	8/8	5/8
	16	α -D-Glucosidase	1/8	8/8	0/8	8/8
ZYM II	17	β -D-Glucosidase	0/8	6/8	8/8	7/8
	4	β -D-Fucosidase	0/8	8/8	4/8	4/8
AP1	9	α -L-Arabinosidase	0/8	8/8	8/8	6/8
	2	L-Pyrrolidone aminopeptidase	8/8	8/8	0/8	8/8
AP2	9	L-Arginine aminopeptidase	1/8	6/8	8/8	7/8
	2	N-benzoyl-L-leucine aminopeptidase	0/8	8/8	1/8	0/8
AP3	3	S-benzyl-L-cysteine aminopeptidase	0/8	0/8	6/8	0/8
	9	L-Tryptophan aminopeptidase	8/8	8/8	7/8	0/8

on the basis of its having a different guanine plus cytosine content in its DNA and a lower homogeneity of DNA, its failure to give cross-transduction of auxotrophic markers (24), and its different α -amylase production (25). In addition to these properties, these authors also described several physiological and biochemical characteristics by which the two species could be separated (24). However, their conclusions were challenged by Gordon et al. (8), who found that the data obtained by Smith et al. (21) did not substantiate this separation. Seki et al. (20), recognizing the difficulty in doing serological studies on the α -amylase produced by strains of *B. subtilis* and *B. amyloliquefaciens* as proposed by Welker and Campbell (25), suggest that at present the homology index by DNA-DNA hybridization and transformability of the auxotrophic markers might be the only effective ways of distinguishing these groups. This study and

that of Baptist et al. (1) indicate that this is no longer the case.

In a previous paper, MacFie et al. (10) reported on the discrimination of low-resolution pyrograms of different genera by using canonical variates analysis and outlined an approach to rapid identification of unknown samples relative to the original canonical variate axes. This paper is concerned primarily in applying PGLC to one particular problem area in *Bacillus* taxonomy, and it has shown that canonical variates analysis can discriminate between pyrograms of species that are phenotypically very similar.

Canonical variates analysis separates groups of points only if there is a sense in which pyrograms of the groups are consistently different. To do this, prior knowledge of the taxonomic structure to be applied is essential. In this study the results of the DNA-DNA hybridizations confirm the validity of the structure applied (i.e.,

TABLE 7. Results of biochemical tests on *Bacillus* strains

Biochemical test	No. of positive strains of:			
	<i>B. subtilis</i>	<i>B. amyloliquefaciens</i>	<i>B. pumilus</i>	<i>B. licheniformis</i>
Catalase	8	8	8	8
Anaerobic growth	0	0	0	8
Voges-Proskauer	8	8	8	8
Egg yolk	2 ^a	0	5 ^b	1 ^c
Growth at pH 5.7	8	8	8	7 ^d
Growth on 5% NaCl	8	8	7 ^e	8
Growth on 7% NaCl	7 ^f	6 ^g	7 ^e	8
Growth on 10% NaCl	4 ^h	4 ⁱ	5 ^j	8
Growth at 50°C	7 ^k	1 ^l	5 ^m	8
Growth at 55°C	0	0	0	6 ⁿ
Acid from:				
Glucose	8	8	8	8
L-Arabinose	6 ^o	2 ^p	8	8
D-Xylose	6 ^o	2 ^p	7 ^q	8
D-Mannitol	6 ^r	8	8	8
Salicin	7 ^s	5 ^t	8	8
Gas from glucose	0	0	0	2 ^u
Hydrolysis of starch	8	8	0	8
Use of citrate	8	8	8	8
Use of propionate	0	0	0	8
NO ₃ → NO ₂	8	8	0	8
Decomposition of casein	8	8	8	8
Decomposition of tyrosine	0	0	0	0

^a Strains 6 and 7 had a weakly opaque zone (diameter, 2 to 4 mm).^b Strains 10, 11, 12, 14 and 16 had a weakly opaque zone (diameter, 2 to 4 mm).^c Strain 7 had a weakly opaque zone (diameter, 2 to 4 mm).^d Strain 18 was negative.^e Strain 13 was negative.^f Strain 6 was negative.^g Strains 27 and 28 were negative.^h Strains 1, 2, 6, and 8 were negative.ⁱ Strains 27, 28, 29, and 30 were negative.^j Strains 10, 12, and 13 were negative.^k Strain 3 was negative.^l Strain 25 was positive.^m Strains 9, 11, and 13 were negative.ⁿ Strains 19 and 24 were negative.^o Strains 2 and 6 were negative.^p Strains 25 and 29 were positive.^q Strain 14 was negative.^r Strains 2 and 3 were negative.^s Strain 2 was negative.^t Strains 28, 31, and 32 were negative.^u Strains 17 and 21 were weakly positive.

four groups), and the discrimination shown by canonical variates analysis illustrates the ability of this approach to mimic taxonomies derived by other methods.

The hybridization results showed that strain 3 was more related to *B. amyloliquefaciens* than to *B. subtilis*. This suggested that this strain was wrongly allocated in the initial canonical variates analysis. However, Fig. 4 does not indicate any obvious difference between this strain and others of the *B. subtilis* group. In canonical variates analysis all of the strains assigned to a group contribute to the variation within the

group and consequently all have an effect on the position of the group mean. To test the allocation of strain 3, it was removed from the data base, and its distance from each of the group means was calculated. These distances showed that strain 3 was more related (closer) to *B. amyloliquefaciens* than to *B. subtilis*. Statistical methods which perform this calculation for all of the strains are available and are being investigated since they should provide valuable information on the stability of the discrimination obtained when canonical variates analysis is used.

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