Rope-Producing Strains of *Bacillus* spp. from Wheat Bread and Strategy for Their Control by Lactic Acid Bacteria

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Two types of white wheat bread (high- and low-type loaves) were investigated for rope spoilage. Thirty of the 56 breads tested developed rope spoilage within 5 days; the high-type loaves were affected by rope spoilage more than the low-type loaves. Sixty-one *Bacillus* strains were isolated from ropy breads and were characterized on the basis of their phenotypic and genotypic traits. All of the isolates were identified as *Bacillus subtilis* by biochemical tests, but molecular assays (randomly amplified polymorphic DNA PCR assay, denaturing gradient gel electrophoresis analysis, and sequencing of the V3 region of 16S ribosomal DNA) revealed greater *Bacillus* species variety in ropy breads. In fact, besides strains of *B. subtilis*, *Bacillus licheniformis*, *Bacillus cereus*, and isolates of *Bacillus clausii* and *Bacillus firmus* were also identified. All of the ropy *Bacillus* isolates exhibited amylase activity, whereas only 32.4% of these isolates were able to produce ropiness in bread slices after treatment at 96°C for 10 min. Strains of lactic acid bacteria previously isolated from sourdough were first selected for antirope activity on bread slices and then used as starters for bread-making experiments. Prevention of growth of approximately 10^4 rope-producing *B. subtilis* G1 spores per cm² on bread slices for more than 15 days was observed when heat-treated cultures of *Lactobacillus plantarum* E5 and *Leuconostoc mesenteroides* A27 were added. Growth of *B. subtilis* G1 occurred after 7 days in breads started with *Saccharomyces cerevisiae* T22, *L. plantarum* E5, and *L. mesenteroides* A27.

Ropiness is bacterial spoilage of bread that initially occurs as an unpleasant fruity odor, followed by enzymatic degradation of the crumb that becomes soft and sticky because of the production of extracellular slimy polysaccharides (15, 43).

The species involved in this type of spoilage of bread are primarily *Bacillus subtilis* and occasionally *Bacillus licheniformis*, *Bacillus pumilus*, and *Bacillus cereus*, even though some rope-producing *Bacillus* isolates are often not identified at the species level (9, 22, 41). It is well known that species differentiation within the genus *Bacillus* is very difficult because of the large number of species and the often incomplete descriptions of a number of newly reported species (19). For these reasons, molecular methods have been used increasingly to simplify characterization procedures in order to provide rapid and reliable identification or to validate phenotypically determined taxa. Among the molecular methods, the randomly amplified polymorphic DNA PCR (RAPD-PCR) assay, 16S ribosomal DNA (rDNA) PCR-denaturing gradient gel electrophoresis (DGGE) analysis, and 16S rDNA sequencing are considered reliable approaches to overcome most of the problems described above. In particular, RAPD-PCR analysis was developed to compare intra- and interspecific differences among *Bacillus* strains (5, 40), and this method has also been shown to be suitable for identifying a variety of *Bacillus* species (32). The second approach, DGGE analysis of the V3 region of 16S rDNA, is a powerful method for differentiating microorganisms at the species level (8, 14), for inferring the phylogenetic

affiliations of community members (25), for evaluating the microbial diversity of several environments (3, 16), and for profiling complex microbial communities (24). However, as far as we know, no previous studies have exploited these techniques to characterize rope-producing *Bacillus* strains from ropy bread.

Spores of *B. subtilis* have been isolated from ropy bread, raw materials, and bakery environments and also from additives, including yeast, bread improvers, and gluten (4, 9, 33, 43). *B. subtilis* spores are heat resistant and can survive during baking in the core of the crumb, where the maximum temperature is 97 to 101°C for a few minutes (31, 33). Ropiness occurs particularly when warm (25 to 30°C) and humid (water activity, ≥ 0.95) environmental conditions allow germination of *Bacillus* spores (46). The water activity, pH, and temperature during storage may also play important roles in spore germination and growth of vegetative cells of *Bacillus* spp. (10, 28, 29). Moreover, rope-causing strains are also characterized by faster development and enhanced protease and amylase production during growth in the bread crumb (31). Current international trends towards elimination of chemical preservatives, including calcium propionate (34, 35), which may cause cancer tumors (34), from bread and other foodstuffs is expected to increase the risk of bread spoilage by rope-producing *Bacillus* strains (4). The traditional sourdough fermentation in bread making allows natural acidification that is able to control rope spoilage. Studies have shown that starting wheat bread with increasing sourdough contents enhances thermal inactivation of *B. subtilis* spores (30). However, although there are many advantages associated with sourdough fermentation in bread making, this process is quite complex and time-consuming compared to the simpler straight dough process.

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The aims of the present study were to investigate the occurrence of rope-producing *Bacillus* spp. in different breads, to characterize the isolates at the phenotypic and genotypic levels, and to determine the antirope activities of selected starter cultures of lactic acid bacteria (LAB) employed in bread making.

MATERIALS AND METHODS

Bread samples. Fifty-six white wheat loaves were obtained from different retail bakers and supermarket stores in southern Italy during a 1-year period. Thirty-two samples of artisanal and industrial high-type breads (height, ≥ 10 cm) and 24 samples of artisanal low-type breads (height, ≤ 10 cm) were studied. The samples were refrigerated at 4°C, transported to the laboratory, and immediately sliced aseptically.

Detection of rope spoilage in bread. Two slices from each loaf were placed into petri dishes (diameter, 150 mm), uniformly soaked with 5 ml of sterile 0.25× Ringer's solution (Oxoid), and incubated at 23 and 30°C. At daily intervals, the slices were examined for rope appearance (sweet rope odor, discoloration of the crumb, and sticky threads).

Isolation and phenotypic characterization of *Bacillus* strains. In order to obtain isolated colonies from ropey breads, the same portions of slimy crumb were streaked on three starch agar (SA) plates (20). After overnight incubation at 30°C, 3 to 10 isolated colonies were picked from the agar plates and purified on SA. Isolates were recognized as *Bacillus* spp. by assessing the presence of spores by phase-contrast microscopy and the Gram reaction as described by Gregersen (18). Identification was performed by using the dichotomous key of Norris et al. (27). Amylase activity was detected by adding drops of an iodine solution (20) to the purified colonies grown on SA. Unhydrolyzed starch formed a blue color with the iodine, while areas of hydrolysis appeared as clear zones resulting from β -amylase activity of the *Bacillus* isolates (20). The isolates were assayed for rope production on sterile bread slices, as described below.

Molecular characterization of *Bacillus* strains. (i) **DNA extraction.** Crude cell extracts were prepared as previously described (23). One microliter of a mixture was used directly as a template for PCR amplification as described below.

(ii) **RAPD-PCR assay and statistical analysis of RAPD profiles.** The RAPD-PCR conditions used with primer XD9 (5' GAAGTCGTCC 3') were the conditions described previously (23). The amplified products (25 μ l) were resolved by electrophoresis on a 1.5% (wt/vol) agarose-Tris-borate gel at 5 V cm^{-1} for 4 h. A 1-kb DNA ladder (Gibco BRL) was used as the molecular weight marker. *B. subtilis* strain ATCC 3366 was used as the reference strain. RAPD fingerprints were normalized with molecular weight markers, and the bands in each RAPD-PCR profile were automatically detected and matched by using the software Phoretic 1, advanced version 3.0.1 (Phoretix International Ltd., Newcastle upon Tyne, England). A correlation matrix for the RAPD patterns of the strains was obtained by using the formula described by Nei and Li (26): $F_{xy} = 2n_{xy}/(n_x + n_y)$, where F_{xy} is the proportion of the reproducible bands that are common to strains x and y , n_{xy} is the number of the reproducible bands shared by strains x and y , and n_x and n_y are the total numbers of reproducible bands obtained for strains x and y , respectively. A similarity matrix was created and used with the average linkage method by using the Cluster procedure of Systat 5.2.1 (42) in order to estimate the percentages of similarity of the RAPD patterns of strains.

(iii) **Amplification of the V3 region of 16S rDNA and DGGE analysis.** Primers V3f (5' CCTACGGGAGGCAGCAG 3') and V3r (5' ATTACCGCGGCTGC TGG 3') spanning the V3 region of the 16S rDNA of *Escherichia coli* (positions 341 to 534) (24) were used for amplification. A GC clamp was added to the forward primer, as described by Muyzer et al. (24). Amplification was performed in a programmable heating incubator (MJ Research Inc., Madison, Wis.). Each mixture (final volume, 25 μ l) contained 1 μ l (about 25 ng) of template DNA, each primer at a concentration of 0.2 μ M, each deoxynucleoside triphosphate at a concentration of 0.25 mM, 2.5 mM MgCl_2 , 2.5 μ l of 10× PCR buffer, and 2.5 U of *Taq* polymerase (Gibco BRL). The template DNA was denatured for 5 min at 94°C. To increase the specificity of amplification and to reduce the formation of spurious by-products, a touchdown PCR was performed (24). The initial annealing temperature used was 10°C above the expected annealing temperature (65°C), and the temperature was decreased by 1°C every second cycle until the touchdown temperature (55°C) was reached; then additional 10 cycles were carried out at 55°C. Primer extension was carried out at 72°C for 3 min. Finally, the samples were incubated for 10 min at 72°C (final extension). The PCR products were analyzed by DGGE by using a Dcode apparatus (Bio-Rad Laboratories, Hercules Calif.). Samples were applied to 8% (wt/vol) polyacrylamide gels in 1× Tris acetate buffer. Parallel electrophoresis experiments were per-

formed at 60°C by using gels containing a 15 to 55% urea-formamide denaturing gradient (100% corresponded to 7 M urea and 40% [wt/vol] formamide) increasing in the direction of electrophoresis. The gels were electrophoresed for 10 min at 50 V and for 3.5 h at 200 V, stained with ethidium bromide for 5 min, and rinsed for 20 min in distilled water.

(iv) **Sequencing of DGGE fragments.** DGGE fragments to be sequenced were excised from the gel with a sterile scalpel and processed with a Qiaex II DNA extraction kit (Qiagen S.p.a., Milan, Italy) according to the suggestions of the manufacturer. Twenty microliters of the eluted DNA from each DGGE band was reamplified by using the conditions described above. Sequences were determined by the dideoxy chain termination method (36) by using a DNA sequencing kit (Perkin-Elmer Cetus, Emeryville, Calif.) according to the manufacturer's instructions and primer V3r.

(v) **Analysis of the sequence data.** To determine the closest known relatives of the partial 16S rDNA sequences obtained, searches were performed by using public data libraries (GenBank [http://www.ncbi.nlm.nih.gov/Blast.cgi]) and the Blast program (1). Similarities were calculated by considering the same length of the sequenced fragments and by using the DNAsis pro. v. 3.0.7 program (Hitachi).

Rope production on bread slices by *Bacillus* strains. Rope-positive *Bacillus* strains isolated from breads were grown overnight at 30°C in 15 ml of bread extract broth (BEB). This medium was prepared by stomaching 350 ml of distilled water and 100 g of commercial sliced white bread for 2 min; the suspension was then filtered through filter paper (Whatman no. 1), and the pH was adjusted to 6.8 with 1 mol of NaOH per liter. The medium was sterilized in tubes at 121°C for 15 min.

The BEB cultures were divided into three 5-ml aliquots. One of the aliquots was heat treated for 10 min at 96°C, one was heat treated for 10 min at 100°C, and the third was used untreated. Each aliquot was homogeneously distributed on slices of autoclaved (121°C, 15 min) commercial slices of carrè white bread to obtain a final concentration of about 10^4 spores cm^{-2} . The slices were incubated at 30°C and examined daily for rope spoilage.

Screening for antagonistic activity of LAB against *B. subtilis* G1. On the basis of slime production, heat resistance, and amylase activity, strain G1 of *B. subtilis* was selected as an indicator to determine the antagonistic activities of 118 homo- and heterofermentative LAB strains. These strains were isolated previously from pizza doughs and baker's yeasts (11, 12), and the numbers of strains belonging to the different taxa are shown in Table 1. Overnight cultures of LAB were assayed by an agar-deferred spot method (45) by using plate count agar (Oxoid SPA, Garbagnate Milanese, Italy) as the culture medium. Inhibition due to H_2O_2 was ascertained by adding catalase (1,000 U/ml) on one side of the spotted cultures. After incubation for 24 h at 30°C, the plates were overlaid with soft SA seeded with about 10^4 CFU of spores of the indicator strain *B. subtilis* G1 per ml. After incubation for an additional 24 h at 30°C, the plates were examined to determine the presence, diameters, and appearance of inhibition zones around the spots.

To ascertain the inhibition due to acidification, a well diffusion assay (37, 45) was used. Overnight cultures of LAB strains grown in appropriate broth media were centrifuged for 10 min at 8,000 $\times g$. The pH values of the cell-free supernatants were adjusted to 7 with 2 mol of NaOH per liter, and the supernatants were filter sterilized (pore size, 0.45 μ m; Sterile Acrodisc; Gelman Science, Ann Arbor, Mich.). Portions (50 μ l) of the supernatant fluids were added to wells (diameter, 6 mm) bored into agar plates containing the indicator strain (about 10^4 CFU g^{-1}). After incubation at 30°C for 24 h, the antimicrobial activity was determined by examining the inhibition zones.

Inhibition of *B. subtilis* G1 on sterile bread slices by heat-treated selected LAB cultures. The LAB strains *Lactobacillus plantarum* E5, *Lactobacillus sanfranciscensis* M207, *Lactobacillus sakei* T56, *Leuconostoc mesenteroides* A27, *Weissella paramesenteroides* A51, and *Enterococcus faecium* A86, which showed the best inhibitory activity against *B. subtilis* G1, were further tested to determine their antirope activities on bread slices. LAB broth cultures that were grown overnight in BEB at 30°C were centrifuged at 2,500 $\times g$ for 10 min, and 5 ml of each supernatant fluid was heat treated at 96°C for 10 min, mixed with a heat-treated suspension of *B. subtilis* G1, and distributed onto sterile bread slices to obtain about 5×10^4 spores/ cm^2 . During incubation at 30°C the slices were examined daily to detect rope slime production and to determine the pH values (2). Slices of bread soaked with a *B. subtilis* G1 spore suspension (5×10^3 spores/ cm^2) were used as a control. The experiment was performed in triplicate.

Bread-making and storage tests. *L. plantarum* E5 and *L. mesenteroides* A27, along with *Saccharomyces cerevisiae* T22, previously isolated from pizza doughs (12), were used as an antirope starter in bread-making and storage tests. All bacterial cultures were propagated in BEB at 30°C for 16 h. The bread doughs were obtained by kneading in a mixer (model KPM50 Professional; KitchenAid, St. Joseph, Mich.) 1,000 g of wheat flour (labeled OO according to the Italian

TABLE 1. Antagonistic activities of LAB against *B. subtilis* G1 determined by the agar deferred spot method

Taxon	No. of strains tested	No. of antagonistic strains	Signature of antagonistic strain(s)	Size of haloes (mm) ^a
<i>Lactobacillus sakei</i>	11	1	T56	5
<i>Lactobacillus plantarum</i>	9	3	E5	6
			E7	3
			T34	3
<i>Lactobacillus paracasei</i>	4			
<i>Lactobacillus pentosus</i>	2	1	E4	4
<i>Lactobacillus confusus</i>	7	1	A38	4
<i>Lactobacillus alimentarius</i>	2			
<i>Lactobacillus rhamnosus</i>	1			
<i>Lactobacillus minor</i>	1			
<i>Lactobacillus sanfranciscensis</i>	1	1	M207	5
<i>Lactobacillus viridiscens</i>	1			
<i>Leuconostoc pseudomesenteroides</i>	15			
<i>Leuconostoc gelidum</i>	13	3	LM47	4
			A43	3
			A72	4
<i>Weissella paramesenteroides</i>	9	1	A51	7
<i>Oenococcus oeni</i>	3			
<i>Leuconostoc mesenteroides</i>	5	1	A27	6
<i>Leuconostoc amelibiosum</i>	2			
<i>Leuconostoc dextranicum</i>	4	1	A90	4
<i>Leuconostoc argentinum</i>	2			
<i>Leuconostoc carnosum</i>	1			
<i>Lactococcus lactis</i> subsp. <i>raffinolactis</i>	1			
<i>Lactococcus garvieae</i>	3			
<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	2			
<i>Lactococcus</i> sp.	18	1	T201	2
<i>Enterococcus faecium</i>	1	1	A86	5

^a The sizes of the haloes are averages from triplicate experiment; the standard deviations were ≥ 0.02 mm and ≤ 0.05 mm.

classification) and 560 g of tap water for 5 min at room temperature. The starter suspension was also added at this stage at a concentration that resulted in viable counts of about 5×10^7 CFU g⁻¹ for both the yeast and LAB in the final doughs. The doughs were artificially contaminated by adding 5×10^4 spores of *B. subtilis* G1 g⁻¹ to the mixing bowl. A preparation inoculated with 5×10^7 CFU of *S. cerevisiae* g⁻¹ and 5×10^4 CFU of *B. subtilis* G1 g⁻¹ was used as a control. After mixing, the doughs were shaped into ca. 400-g loaves, placed in aluminum pans, and leavened at 30°C until the volume was twice the initial volume. The leavened doughs were cooked in an oven at 250°C for 20 min. After cooling, the treated breads and the control were stored at 23 and 30°C. For 7 days the loaves were examined externally for the presence of the typical sweet fruity odor which

characterizes the first stage of ropiness. The breads that gave off an intense smell were cut into two parts to examine the internal loaf crumb. The pH and total titratable acidity (2) were measured at the end of the incubation period.

Nucleotide sequence accession numbers. The GenBank accession numbers for 16S rDNA partial sequences retrieved from DGGE bands are shown in Table 2.

RESULTS

Detection of ropy breads. A total of 53.6% of the loaves examined showed typical rope production; 63% of the rope-

TABLE 2. RAPD-PCR and DGGE patterns and molecular identification of some *Bacillus* spp. strains

RAPD-PCR pattern	Strain(s) ^a	V3 16S rDNA DGGE pattern	Accession no.	Identification by V3 16S rDNA sequencing (most closely related species)
A	A3, S5, Q1	a	AY033067	<i>B. cereus</i> (99%), <i>B. anthracis</i> (99%), <i>B. thuringiensis</i> (99%) ^b
B	R1	b	AY056042	<i>B. clausii</i> (95%)
C	A1, B1, C3, D1, G1, H1, L2	c	AY033068	<i>B. subtilis</i> (99%), <i>B. vallismortis</i> (99%)
D	A2, E1, G3	a	AY033070	<i>B. cereus</i> (99%), <i>B. anthracis</i> (99%), <i>B. thuringiensis</i> (99%)
E	S6, S9, V1g	c	AY033069	<i>B. subtilis</i> (98%), <i>B. licheniformis</i> (98%)
F	U1, V1p	d	AY033071	<i>B. firmus</i> (97%)
G	K2, N1, O1, P2, S1, X1, Z1	b	AY056043	<i>B. clausii</i> (98%)
H	ATCC 9799	c	AF142574 ^c	<i>B. subtilis</i>
I	U2, I1, P5,	c	AY056044	<i>B. subtilis</i> (98%), <i>B. licheniformis</i> (98%)
J	F1, J1, T1	c	AY056046	<i>B. subtilis</i> (98%)
K	M1p, Y1	a	AY056045	<i>B. cereus</i> (99%), <i>B. anthracis</i> (99%), <i>B. thuringiensis</i> (99%)

^a Strains A3, R1, G1, G3, S6, V1p, K2, U2, F1, and M1p were identified by sequencing the V3 region of the 16S rDNA.

^b The values in parentheses are levels of similarity, which were calculated by considering the same length of sequenced fragments and by using the DNAsis pro. v. 3.0.7 program (Hitachi) and public data libraries (GenBank [http://www.ncbi.nlm.nih.gov/Blast.cgi]) (1).

^c *B. subtilis* ATCC 9799 (17).

FIG. 1. Cumulative total numbers of rope-spoiled breads during storage at 23 and 30°C. A total of 56 samples were analyzed.

positive samples were high-type breads, whereas 37% were low-type breads. As shown in Fig. 1 the majority of the bread samples exhibited rope symptoms after 2 or 3 days of storage at 23 and 30°C, although the higher temperature (30°C) resulted in faster development of ropiness (1 to 2 days). No other ropy breads were detected between days 5 and 15 of storage at either 23 or 30°C.

Characterization of *Bacillus* isolates. (i) Isolation and phenotypic characterization. A total of 61 cultures of gram-positive spore-forming rods were isolated from the 30 ropy bread samples. All the isolates were identified as *B. subtilis* by using the dichotomous key of Norris et al. (27).

The *B. subtilis* isolates were characterized to determine their rope activity, resistance to heating, and amylase activity. As shown in Table 3, 37 (66%) of the 61 strains assayed without heat treatment were able to cause ropiness in sterile bread slices. When the *Bacillus* strains were heat treated for 10 min at 96°C, only 12 (32.4%) of them still produced ropiness. No ropiness was observed when the strains were treated at 100°C for 10 min. Moreover, all rope-positive *Bacillus* isolates were able to hydrolyze the SA to dextrin and maltose (Table 3). Among the heat-resistant rope-producing *Bacillus* isolates, strain G1 produced the largest zone of amylase activity (Table 3) and hence was chosen as the indicator strain for the subsequent experiments.

(ii) Molecular characterization. Thirty-four strains of *B. subtilis* were chosen on the basis of their origins (isolation samples) and their biochemical activities and were characterized by molecular methods. These strains showed 11 different

patterns after RAPD-PCR analysis (Fig. 2). As shown in Table 2, most RAPD-PCR patterns were obtained for more than one strain; the only exceptions were patterns B and H, which were obtained only for strain R1 and *B. subtilis* ATCC 9799, respectively. In most cases, a RAPD-PCR pattern was produced by strains isolated from different samples. Nevertheless, the levels of similarity among RAPD-PCR patterns were very low, ranging from 20 to 60% (data not shown).

Sequence-specific separation of the V3 region of 16S rDNA by DGGE resulted in grouping of the 34 strains in four profiles (Fig. 3). As shown in Table 2, the DGGE profile a group contained seven strains that produced three different RAPD-PCR patterns (patterns A, D, and K); the DGGE profile b group contained eight strains that produced two RAPD-PCR patterns (patterns B and G); the DGGE profile c group contained 17 strains, including *B. subtilis* ATCC 9799, that produced RAPD-PCR patterns C, E, H, I, and J; and the DGGE profile d group contained two strains that produced RAPD-PCR pattern F.

Molecular identification of 10 strains, representing both DGGE and RAPD-PCR patterns, was performed by sequencing the V3 region of 16S rDNA (*E. coli* positions 341 to 534). The accession numbers for the sequences retrieved are shown in Table 2. Strains that produced DGGE pattern a were most closely related (level of similarity, 99%) to *B. cereus*, *Bacillus thuringiensis*, and *Bacillus anthracis*; strains that produced DGGE pattern b were most closely related (95 to 98%) to *Bacillus clausii*; strains that produced DGGE pattern c were most closely related (98 to 99%) to *B. subtilis*, *B. licheniformis*,

TABLE 3. Rope formation and amylase activities of the *B. subtilis* strains isolated from ropy bread

Source		Strain(s)	Isolation temp ^b		Rope activity after heat treatment ^c			Amylase activity ^d
Sample	Type ^a		23°C	30°C	96°C	100°C	Untreated	
A	h	A1, A4	+		—	—	+	+
		A2	+		—	—	—	+
		A3	+		—	—	—	—
		A5, A6		+	—	—	+	+
B	h	B1	+		—	—	+	+
		B2, B3		+	—	—	+	+
C	h	C1	+		—	—	—	—
		C3, C6	+		+	—	+	++
		C2, C4, C5, C7		+	—	—	—	—
D	h	D1		+	—	—	+	+
E	l	E1		+	—	—	+	+
F	h	F1		+	—	—	+	+
G	h	G1		+	+	—	+	+++
		G2, G4		+	—	—	+	++
		G3, G5		+	—	—	—	—
		H1		+	+	—	+	++
I	h	I1		+	—	—	—	—
J	h	J1		+	—	—	+	++
		J2	+		—	—	+	+
K	h	K1, K2		+	+	—	+	++
L	h	L1, L2		+	—	—	+	+
M	h	M1p		+	—	—	+	+
N	h	N1	+		+	—	+	++
O	h	O1	+		+	—	+	+
		P1	+		—	—	+	+
		P2	+		+	—	+	+
		P5	+		—	—	—	—
Q	h	P3, P4, P6		+	—	—	—	—
		Q1	+		—	—	—	—
		Q2		+	—	—	—	—
R	l	R1		+	—	—	—	—
S	l	S1	+		—	—	+	+
		S5, S14		+	—	—	—	—
		S6, S9, S17		+	+	—	+	++
		S12, S13, S19		+	—	—	+	++
T	h	T1		+	—	—	+	+
U	l	U1		+	—	—	+	+
		U2		+	—	—	—	—
V	h	V1p, V1g		+	—	—	+	+
X	h	X1	+		—	—	+	+
Y	h	Y1		+	—	—	+	+
Z	h	Z1		+	—	—	+	+

^a The types of loaves tested with artisanal and industrially made high-type breads (h) artisanal low-type breads (l).

^b Bread samples were incubated at 23 and 30°C.

^c Broth cultures of *B. subtilis* strains were heat treated at 96 and 100°C for 10 min before the rope production assay on sterile carré white bread slices. Untreated broth cultures were used as controls.

^d Amylase activity was determined by the staining method on SA. Halo diameters: +++, >10mm; ++, 5 to 10 mm; +, <5 mm—, no activity.

and *Bacillus vallismortis*; and finally, strains that produced DGGE pattern d were most closely related (97%) to *Bacillus firmus*.

Activity of LAB against *B. subtilis* G1 as determined by the agar well diffusion method. The antirope activities of LAB, as shown in Table 4, were demonstrated by an agar spot deferred antagonism method and were confirmed by an agar well diffusion assay performed with neutralized and filtered culture supernatants. *B. subtilis* G1, selected on the basis of its heat resistance and amylase activity as described above, was used as the indicator strain. The possibility of interference of hydrogen peroxide production was eliminated by adding catalase (1,000 U/ml) on one side of the spotted cultures. As shown in Table 1, 15 (12.7%) of 118 LAB strains assayed produced zones of inhibition of *B. subtilis* G1. The largest inhibition haloes (di-

ameters, 5 to 7 mm) were produced by *L. sakei* T56, *L. plantarum* E5, *L. sanfranciscensis* M207, *W. paramesenteroides* A51, *L. mesenteroides* A27, and *E. faecium* A86. The inhibition zones disappeared when neutralized cell-free supernatants were assayed; therefore, the antimicrobial activity was due to acidification.

Inhibition of *B. subtilis* G1 by LAB on bread slices. The inhibitory activities of heat-treated supernatants of the LAB strains selected as described above were tested by using sterile bread slices and *B. subtilis* G1 (Table 4). *L. plantarum* E5 and *L. mesenteroides* A27 inhibited ropiness in the bread for more than 15 days, whereas the other strains were able to inhibit the growth of *B. subtilis* G1 for a minimum of 2 days and a maximum of 5 days during incubation at 30°C. The pH decreased from 4.9 at the beginning of storage (data not shown) to values

FIG. 2. Ethidium bromide-stained 1.5% (wt/vol) agarose gel of RAPD-PCR products of 13 *Bacillus* spp. isolates generated by using primer XD9. Lane 1, strain G3 (pattern D); lane 2, strain F1 (pattern J); lane 3, strain G1 (pattern C); lane 4, strain M1p (pattern K); lane 5, strain U2 (pattern I); lane 6, strain R1 (pattern B); lane 7, strain A3 (pattern A); lanes 8 and 13, strain ATCC 9799 (pattern H); lane 9, strain S6 (pattern E); lane 10, strain V1p (pattern F); lane 11, strain K2 (pattern G); lane 12, strain A1 (pattern C); lanes M, 1-kb DNA ladder (Gibco BRL) used as a molecular weight marker.

ranging from 4.3 to 3.7 during the inhibition period (Table 4). The development of rope in the bread slices caused a rapid increase in the pH to 5. The control slices inoculated with *B. subtilis* G1 spores without LAB were ropy after overnight incubation (Table 4).

Antirope activity in baked bread. *L. plantarum* E5 and *L. mesenteroides* A27 were used as components of the antirope starter in the bread-making experiments. In order to obtain leavening, the starter was complemented with *S. cerevisiae* T22. The preservative starter delayed rope spoilage of bread for 1

FIG. 3. Sequence-specific DGGE separation of the V3 regions of 16S rDNA of 14 *Bacillus* spp. strains. Lane 1, strain R1 (pattern b); lane 2, strain G1 (pattern c); lane 3, strain A3 (pattern a); lane 4, strain K2 (pattern b); lane 5, strain G3 (pattern a); lane 6, strain ATCC 9799 (pattern c); lane 7, strain V1p (pattern d); lane 8, strain U2 (pattern c); lane 9, strain F1 (pattern c); lane 10, strain U1 (pattern d); lane 11, strain S1 (pattern b); lane 12, strain B1 (pattern c); lane 13, strain Z1 (pattern b); lane 14, strain M1p (pattern a).

TABLE 4. Antirope activities of LAB with *B. subtilis* G1, detected on sterile bread slices during storage at 30°C for 15 days^a

Strain	Time to rope symptoms (days)	pH of bread slices ^b
<i>Lactobacillus plantarum</i> E5	NR ^c	3.7 ± 0.10
<i>Lactobacillus sanfranciscensis</i> M207	3	4.0 ± 0.20
<i>Lactobacillus sakei</i> T56	3	3.9 ± 0.20
<i>Leuconostoc mesenteroides</i> A27	NR	3.8 ± 0.10
<i>Weissella paramesenteroides</i> A51	5	3.9 ± 0.15
<i>Enterococcus faecium</i> A86	2	4.3 ± 0.20
<i>Bacillus subtilis</i> G1 (positive control)	0.5	ND ^d

^a Heat-treated (96°C, 10 min) supernatants from LAB cultures were used in the experiment.

^b The pH values are the values measured on the day before the rope symptoms appeared.

^c NR, no rope.

^d ND, not determined.

week when the bread was stored at 23°C (Fig. 4A). Incubation of the bread at 30°C resulted in development of rope after 4 days of storage (data not shown). Bread started with *S. cerevisiae* alone exhibited ropiness after overnight incubation at both 23 and 30°C (Fig. 4B). The pH values of the loaves of bread

FIG. 4. Rope production in bread started with *S. cerevisiae* T22 after 1 day of storage at 23°C (A) and rope production in bread started with *L. plantarum* E5, *L. mesenteroides* A27, and *S. cerevisiae* T22 after 7 days of storage at 23°C (B). The breads were artificially contaminated with *B. subtilis* G1.

prepared with and without the antirope starter were 4.6 and 5.5, respectively, whereas the total titratable acidity values were 1.8 and 0.7 ml of 0.1 N NaOH 10 g^{-1} , respectively (data not shown).

DISCUSSION

Detection of rope spoilage in bread. According to previous studies (4, 15, 44), ropy samples develop a typical fruity odor after 12 to 24 h of incubation at 23 and 30°C, whereas the crumb becomes stickier and softer during the following days. More than one-half of the breads tested developed rope in 5 days; however, high-type breads were more frequently affected by ropiness than low-type breads. These results demonstrate that failure of the baking process is expected especially in high-type bread, in which heat-resistant *Bacillus* spores can survive in the center of the crumb, where the temperature often is not 96 to 101°C for 10 min (33, 38).

Identification and characterization of *Bacillus* strains. Detection of rope spoilage, performed directly with bread samples, allowed isolation of different strains of spoilage-causing bacteria. Identification and differentiation of *Bacillus* spp. isolates from bakery environments on the basis of their phenotypic traits are very difficult (9). Collins et al. (9) suggested that additional methods, such as numerical taxonomy with combined results, can be used to clarify discrepancies in the identification of isolates. In this work a polyphasic approach, in which both biochemical and molecular methods were used, was used to characterize the *Bacillus* strains isolated from ropy bread. In some cases, the identities obtained by using biochemical and molecular assays differed considerably. Using the key of Norris et al. (27) resulted in identification of all of the isolates as *B. subtilis*, which, according to Rosenkvist and Hansen (33), could be considered the only species associated with ropiness in bread. In this study, molecular identification resulted in a greater variety of species than phenotypic characterization. In fact, strains were grouped into 11 biotypes with low degrees of similarity (<62%) as a result of using the RAPD-PCR technique. On the basis of the electrophoretic mobility of the 16S rDNA V3 region in DGGE gels, these biotypes were further clustered into four groups, suggesting the presence of at least four different species according to Muyzer et al. (24). Furthermore, on the basis of a 16S rDNA V3 region sequence analysis of representative strains of the two groups obtained by the molecular techniques mentioned above, it was possible to identify the following four taxonomic groups: (i) a *B. cereus* group (including *B. cereus*, *B. anthracis*, and *B. thuringiensis*) (group a); (ii) *B. clausii* (group b); (iii) a *B. subtilis* group (including *B. subtilis*, *B. licheniformis*, and *B. vallismortis*) (group c); and (iv) *B. firmus* (group d). Unfortunately, due to the high levels of sequence similarity of the 16S rDNA V3 regions of *B. cereus*, *B. anthracis*, and *B. thuringiensis* and the high levels of sequence similarity of the 16S rDNA V3 regions of *B. subtilis*, *B. licheniformis*, and *B. vallismortis* (data not shown), it was impossible to identify the strains of taxonomic groups a and c at the species level.

The molecular approach used in this study can therefore be considered valid for detecting the occurrence of species with unequivocal DGGE profiles and useful for confirming or facilitating taxonomic identification of *Bacillus* cultures. The lat-

ter use is enhanced by knowledge of additional cultural or biochemical traits. In the case of species belonging to groups whose members have similar or identical DGGE profiles, identification is facilitated by the possibility of selecting a specific biochemical assay or species-specific PCR primers, as suggested by Walters et al. (48), which distinguish a smaller number of candidate taxa.

Hence, further investigations involving other 16S rDNA regions or other target genes are necessary in order to find fragments that result in specific DGGE profiles. Indeed, recently, the 16S rDNA V1 region and the *gyrA* (gyrase A) gene have been evaluated as potential species-specific markers for the *B. subtilis* and *B. cereus* groups (6, 7).

The taxonomic profile obtained by the molecular methods used in this study appeared to be similar to the profiles detected by Collins et al. (9), Bailey and von Holy (4), and Thompson et al. (43), even if some differences were found. Strains of *B. subtilis*, *B. licheniformis*, *B. cereus*, and *B. firmus* are generally recognized as contaminants in bread manufacturing (4, 9, 43). However, we also identified other isolates obtained from spoiled bread, such as *B. clausii*. Strains of *B. firmus* were previously isolated only from raw materials and wheat grains (9, 33) and, along with *B. clausii*, were able to cause ropiness in bread. Some authors (4, 9, 21, 33) consider the possibility of high levels of contamination by *B. cereus* in bread remote, while other studies have documented cases of food poisoning involving nausea, vomiting, diarrhea, headaches, and chills associated with the consumption of ropy bread (22, 44, 47). However, the presence of rope-producing *B. cereus* in bread has to be considered a factor that could result in food poisoning and suggests that contamination should be prevented.

All of the strains isolated were also characterized to determine their amylase activities and heat resistance. The relationship between ropiness and hydrolysis of starch by microbial amylases, as reported previously by Fisher and Halton (15), Kirschner and von Holy (22), and Rosenkvist and Hansen (33), was confirmed by our findings, since all rope-positive isolates of *Bacillus* showed amylase activity on SA.

Rope production by heat-treated broth cultures of *Bacillus* strains was determined by using sterilized bread slices. Heat treatments greatly decreased the number of strains able to cause rope in bread slices. In agreement with other studies (4, 33), in which isolated *B. subtilis* strains survived the baking process, most of the *Bacillus* strains detected in this study were resistant to heating at 96°C for 10 min. Moreover, our experiment also revealed rope activity by heat-resistant strains of *B. clausii*, a new species related to rope spoilage in bread. These findings highlight the danger of underbaking bread, which might result in increasing the number of *Bacillus* species that survive the baking process, thus reducing the product shelf life.

Inhibitory activity of LAB against *B. subtilis* G1. Antirope activity has to be considered an important characteristic for selecting LAB able to extend the shelf life of baked products. Only 12.7% of the LAB strains assayed for antagonistic activity showed inhibitory activity against *B. subtilis* G1. As shown by a well diffusion assay, the inhibition of *B. subtilis* G1 was due to the acidity of the broth supernatants obtained from LAB cultures. Bacteriocins have been found to confer antagonistic

potential to LAB isolates from dough (13, 34). However, we did not detect any bacteriocin-like substance produced by the LAB strains used.

The inhibitory effects of LAB strains (*L. sakei* T56, *L. plantarum* E5, *L. sanfranciscensis* M207, *W. paramesenteroides* A51, *L. mesenteroides* A27, and *E. faecium* A86) on *B. subtilis* G1 were evaluated by testing supernatant fluids heated at 96°C for 10 min on bread slices. With this treatment we aimed to select LAB strains under conditions resembling those during the baking process. *L. plantarum* E5 and *L. mesenteroides* A27 showed the most effective antirope activity, inhibiting the development of ropiness for more than 15 days. Many authors (28, 29, 41) have described the importance of pH as a controlling factor in the development of ropiness. According to our observations, the time of inhibition of rope symptoms increases at a low pH (range, pH 3.7 to 4.3). This finding differs from the observations of other authors that indicated that the rope-inhibiting pH values were 4.69 (39) and 4.8 (34). The differences may have been due to the different experimental conditions and the different strains used as indicators.

The screening steps used in this work were useful for selecting the most effective antirope LAB strains for use in bread-making and storage tests. This approach was chosen based on the results obtained by authors who studied the activities of LAB strains against *Bacillus* in agar media or in bread. As reported by Rosenquist and Hansen (34), cultures of bacteriocin-producing LAB added as starters in wheat dough had no effect on *B. subtilis* and *B. licheniformis* strains, despite the fact that the same nisin-producing LAB strains had shown inhibitory activity against *B. subtilis* and *B. licheniformis* in an agar spot assay. This suggested that screening LAB by using conventional laboratory media might provide results that differ from the real activities of the strains selected. It might be advisable to select antirope starter cultures by using a medium with a composition very similar to that of bread. In our study this objective was achieved by growing the antagonistic LAB in BEB, a natural medium obtained by bread extraction, followed by an antagonistic assay with *B. subtilis* G1 directly on bread slices.

Antirope activity in baked bread. The possible use of microbial cultures to increase the shelf life and safety of food products requires development of techniques which demonstrate specific inhibitory activities by starters directly in food manufacturing. Compared to bread started with *S. cerevisiae* T22 alone, bread manufactured with *L. plantarum* E5 and *L. mesenteroides* A27 associated with *S. cerevisiae* T22 delayed rope symptoms for a time that depended on the storage temperature used. A lower storage temperature (23°C) extended the bread shelf life for 7 days, whereas storage at 30°C controlled ropiness for 4 days. Storage of bread at temperatures below 25°C, which has been indicated by some authors to be an important controlling factor (28), has to be associated with the antirope activity of the starter in order to prevent the growth of *Bacillus* and prolong the shelf life of the bread.

The high percentages of ropy breads detected in this study and the climatic conditions of southern Italy, which favor growth of the bacteria, are expected to increase the risk of spoilage caused by *Bacillus* in artisanal and industrially baked products. Phenotypic identification and molecular identification highlight the fact that together with *B. subtilis* and *B.*

licheniformis, which are always related to rope activity, other *Bacillus* species might be able to cause ropiness in bread. Rope spoilage cannot be completely prevented by using only the recommended control procedures, such as analysis of the raw material, controlling the hygiene of the bakery equipment, and strict temperature control during baking. The use of an anti-rope starter consisting of selected LAB strains may be considered a natural and effective method to prevent rope spoilage in bread.

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