RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY (2011) 14:213-223
DOI: 10.2436/20.1501.01.151 ISSN: 1139-6709 **www.im.microbios.org**

INTERNATIONAL MICROBIOLOGY

Antimicrobial peptide genes in *Bacillus* strains from plant environments

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Received 10 October 2011 · Accepted 17 November 2011

Summary. The presence of the antimicrobial peptide (AMP) biosynthetic genes *srfAA* (surfactin), *bacA* (bacylisin), *fenD* (fengycin), *bmyB* (bacyllomicin), *spaS* (subtilin), and *ituC* (iturin) was examined in 184 isolates of *Bacillus* spp. obtained from plant environments (aerial, rhizosphere, soil) in the Mediterranean land area of Spain. Most strains had between two and four AMP genes whereas strains with five genes were seldom detected and none of the strains had six genes. The most frequent AMP gene markers were *srfAA, bacA, bmyB*, and *fenD*, and the most frequent genotypes *srfAA-bacA-bmyB* and *srfAA-bacA-bmyB-fenD.* The dominance of these particular genes in *Bacillus* strains associated with plants reinforces the competitive role of surfactin, bacyllomicin, fengycin, and bacilysin in the fitness of strains in natural environments. The use of these AMP gene markers may assist in the selection of putative biological control agents of plant pathogens. [**Int Microbiol** 2011; 14(4):213-223]

Keywords: *Bacillus* · antimicrobial peptides · bacillomycin · fengycin · surfactin · bacilysin

Introduction

Plant disease control has been reoriented towards the rational use of fungicides and bactericides and the application of non-chemical methods with decreased environmental impact. Microbial biopesticides, which consist of microbial strains including bacterial or fungal species and bacteriophages [37], offer an alternative to or are able to complement chemical pesticides [19,35,40]. For example, several strains of the bacteria *Pantoea agglomerans* [18] in addition to *Pseudomonas fluorescens* [51], *Pseudomonas chlororaphis* [53], *Lactobacillus* spp. [54] and *Bacillus subtilis* [34] have been reported in the successful control of many plant diseases.

Bacillus subtilis and related species have been the object of particular interest because of their safety, their widespread distribution in very diverse habitats, their remarkable ability to survive adverse conditions due to the development of endospores, and their production of compounds that are beneficial for agronomical purposes [16,17,25,34,37,39]. Several strains of *Bacillus* have been shown to control plant diseases by different mechanisms of action, including antibiosis, the induction of defense responses in the host plant, and competition for nutrient sources and space [2,14,42,46]. Among these mechanisms, antibiosis by means of antimicrobial peptides has been explored in detail [39].

Antimicrobial peptides (AMPs) produced by *Bacillus* spp. have been implicated in the biocontrol of several plant pathogens causing aerial, soil, and postharvest diseases [4,10,12,17,23,24,28,32,36,50] and in the promotion of plant

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growth [22,25,45]. AMPs include cyclic lipopeptides such as fengycin, iturin, bacillomycin, and surfactin. These compounds are characterized by a wide antimicrobial spectrum and intense surfactant activities [50,55], and they have been implicated in the biocontrol of several plant diseases [6,9,12,38,46]. In addition to lipopeptides, other peptidic compounds are active in the biocontrol of plant pathogens, such as bacilysin, a dipeptide described in *B. amyloliquefaciens* FZB42 [11,41,43], and subtilin, a lantibiotic described in *B. subtilis* [8,29,49]. Note that, in several strains of *Bacillus,* the biocontrol of plant pathogens has been linked to the presence of the AMP biosynthetic genes *bmyB*, *fenD*, *ituC*, *srfAA*, and *srfAB* [20,23,46]. The simultaneous production of different AMPs is important for the efficiency of disease control and underlies the broad range of antagonistic activity in *Bacillus*. Specifically, the production of mixtures of bacillomycin, fengycin, and iturin A by *B. subtilis* has been related to the control of *Podosphaera fusca* in cucurbits [46], and the production of bacilysin, iturin, and mersacidin in *B. subtilis* ME488 to the suppression of *Fusarium* wilt of cucumber and *Phytophthora* blight of pepper [12]. Accordingly, strains of *Bacillus* that score positive for all of the abovementioned AMP biosynthetic genes are more effective at inhibiting the growth of *Rhizoctonia solani* and *Pythium ultimum* than other *Bacillus* isolates that lack one or more of those markers [23]. In addition, genome analysis of the commercial strain *B. amyloliquefaciens* FZB42 revealed the presence of genes responsible for the synthesis of several antimicrobial compounds [11], and similar genes have been reported in the commercialized *B. subtilis* strains GB03, QST713, and MBI600 [3,23].

An understanding of the role of AMPs in the ecological fitness of plants requires an appreciation of the distribution of the respective genes in natural populations of the relevant *Bacillus* species. However, thus far, our knowledge is restricted to strains identified based on their antagonism of specific target plant pathogens such as *Sclerotina sclerotiorum* [5] or other fungal pathogens of canola and wheat [44]. By contrast, little is known about the distribution and, specifically, the number and expression pattern of AMP biosynthetic genes in strains of *Bacillus* not previously selected for antagonism against a given plant pathogen.

Thus, the aim of this work was to study the distribution of six AMP genes (*srfAA*, surfactin; *bacA*, bacylisin; *fenD,* fengycin; *bmyB*, bacyllomicin; *spaS*, subtilin; and *ituC*, iturin) in an unselected collection of *Bacillus* spp. obtained from different plant environments in a Mediterranean land area.

Materials and methods

Bacterial strains and growth conditions. A collection of reference strains of *Bacillus* including 14 strains belonging to six species of the genus was used. The strains were *B. amyloliquefaciens* FZB42 (ABiTEP GmbH, Germany), *B. subtilis* QST713 (Agraquest, USA), *B. subtilis* EPS2004 (CIDSAV, University of Girona, Spain), *B. subtilis* UMAF6614 and UMAF6639 (Departament of Microbiology, University of Malaga, Spain), *B. subtilis* RGAF9, RGAF32, *B. circulans* RGAF11, *B. polymyxa* RGAF5 and RGAF84, *B. macerans* RGAF101, *B. megaterium* RGAF46 and RGAF51, and *Bacillus* RGAF66 (Institute for Sustainable Agriculture, CSIC, Spain). In addition, several non-*Bacillus* bacterial species were examined: *Pantoea agglomerans* EPS10, EPS13, EPS21, EPS130, EPS132, EPS156, EPS207, EPS210, EPS230, and EPS453, *Pseudomonas fluorescens* ESP62e, EPS82, EPS87, EPS95, EPS98F, EPS102, EPS173, ES282, ESP353, and EPS684, *Pseudomonas syringae* EPS94 and *Serratia marcescens* EPS131 and EPS318 (CIDSAV, University of Girona, Spain), *Pectobacterium carotovorum* CECT225 (Spanish Type Culture Collection, Valencia, Spain), *Xanthomonas arboricola* pv. fragariae CFBP3549 and *Xanthomonas axonopodis* pv. vesicatoria CFBP327 (CFBP, French Collection of Plant Pathogenic Bacteria, UMR PaVé-INRA, France), and *Pseudomonas syringae* pv. tomato DC3000 (NCPPB4369) (NCPPB, National Collection of Plant Pathogenic Bacteria, UK).

Bacillus strains were cultured in Luria-Bertani (LB) agar at 28 ºC for 24 h, and *P. agglomerans*, *P. fluorescens*, and *S. marcescens* in LB at 23 ºC also for 24 h. Concentrations of cell suspensions for each bacterial genus were obtained using a standard curve relating cell concentration to optical density at 620 nm.

DNA extraction. DNA was obtained according to the method described by Llop et al. [31]. Briefly, 1 ml of bacterial suspension was centrifuged at 10,000 ×*g* for 10 min and the pellet was resuspended in 500 µl of extraction buffer (200 mM Tris-HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA, 05 % SDS, 2 % PVP)**.** After 1 h of shaking, the tubes were centrifuged at 5000 ×*g* for 5 min and 450 µl was transferred to a new tube to which 450 µl of isopropanol was then added. Precipitation took place over at least 1 h, followed by centrifugation at 13,000 ×*g* for 10 min. The DNA pellet was dried and then resuspended in 200 µl of sterile ultrapure water.

AMP gene primer design and PCR assays. For the development of PCR primers, six sequences were chosen from the coding regions of *bmyB* (bacillomycin L synthetase B), *fenD* (fengycin synthetase), *ituC* (iturin A synthetase C), *srfAA* (surfactin synthetase subunit 1), *bacA* (bacilysin biosynthesis protein), and *spaS* (lantibiotic subtilin). Also, two sequences were used as markers of *Bacillus*, based on the coding regions of the 16S rRNA genes and *spoVG* (putative septation protein spoVG). Primers reported by Joshi and McSpadden [23], to detect the genes *bmyB*, *fenD*, *ituC*, and *srfAA*, were used for comparison. New primers were developed for *bmyB*, *fenD*, *ituC*, and *srfAA* due to the need to increase the sensitivity of the PCR, which is essential when working with natural samples. Gene sequences were obtained from the GenBanK database and aligned using MultAlin software [13]. Consensus regions were used to design specific primers using Primer3 software [47] (Table 1). PCR was carried out in a total volume of 50 µl containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP (Invitrogen Technologies), 0.2 µM of each primer, 2.0 U of Taq DNA polymerase (Biotools), and 4 µl of genomic DNA. The cycling conditions for the amplification of all targets were as follows: 95 ºC for 4 min, 40 cycles of 94 ºC for 1 min, annealing temperature for 1 min, and 70 ºC for 1 min. A final extension step at 70 ºC for 5 min was followed by a 4 ºC soak. The annealing temperature was set to 58 ºC for 16S rRNA genes and for *fenD*, *ituC*, *srfAA*,

Table 1. Oligonucleotide primers used to detect genetic markers in field samples and in *Bacillus* spp. strains

Primer	Expression product	Sequence (5′ → 3′)	Gene	Melting T (ºC)	Product size (bp)
16SBACF	16S rRNA	GCTTGCTCCCTGATGTTAGC	*16S rDNA*	59.9	163
16SBACR		CGGGTCCATCTGTAAGTGGT			
SPOF	Spore protein	AATACCGATGGTCGCATGA	*spoVG*	59.5	226
SPOR		CAGAATCACCCAAACGATGA			
FENDF	Fengycin	GGCCCGTTCTCTAAATCCAT	*fenD*	60.1	269
FENDR		GTCATGCTGACGAGAGCAAA			
BMYBF	Bacyllomicin	GAATCCCGTTGTTCTCCAAA	*bmyB*	59.9	370
BMYBR		GCGGGTATTGAATGCTTGTT			
ITUCF	Iturin	GGCTGCTGCAGATGCTTTAT	*ituC*	60.1	423
ITUCR		TCGCAGATAATCGCAGTGAG			
SRFAF	Surfactin	TCGGGACAGGAAGACATCAT	*srfAA*	60.4	201
SRFAR		CCACTCAAACGGATAATCCTGA			
BACF	Bacylisin	CAGCTCATGGGAATGCTTTT	*bacA*	60.1	498
BACR		CTCGGTCCTGAAGGGACAAG			
SPASF	Subtilin	GGTTTGTTGGATGGAGCTGT	*spaS*	59.6	375
SPASR		GCAAGGAGTCAGAGCAAGGT			

bacA and *spaS*, to 55 ºC for *bmyB*, and to 52 ºC for *spoVG*. Amplifications were carried out in a T3000 thermocycler (Biometra, Germany). The amplification products were analyzed in a 1.8 % agarose gel in 1× Tris-acetate EDTA (TAE), run for 45 min at 90 V, and viewed after staining with ethidium bromide. Size comparisons were made with a 1-kb plus ladder (Invitrogen, California, USA). Gel images were captured with an imaging system (Kodak 120; Kodak, Rochester, NY, USA).

The sensitivity of the primer pairs was determined in four *Bacillus* strains (RGAF51, UMA6614, UMA6639, and EPS2004). Suspensions of each strain were prepared at 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 colony-forming units (CFU)/ml and DNA extracted as described above. The specificity of the primer pairs was determined as described above in 14 strains of *Bacillus* spp., 10 strains of *P. agglomerans*, 10 strains of *P. fluorescens*, and 2 strains of *S. marcescens*. Suspensions of each strain were prepared at 10^6 and 10^8 CFU/ml and DNA was extracted as before.

Sampling and isolation procedures. Field samples from plant environments were collected from three locations of the Mediterranean area of Spain in late spring–early summer (Table 2). For validation of the selective enrichment method, 45 samples were collected, mainly from Girona, Lleida, and Menorca. Fifteen samples were from the aerial plant part, 15 from the rhizosphere, and 15 from the soil sorrounding the plant root system. The sampled plants were representative species of ten families of herbaceous plants typical of the Mediterranean area. To compare the efficiency of isolation of the *Bacillus* strains, two methods were compared: the standard method (ST) and the selective enrichment method (SE). The ST method was based on the recovery of *Bacillus* isolates directly from the sample extract and was carried out as follows: 1 g of material was homogenized for 90 s in 10 ml of phosphate buffer (0.02 M Na_2HPO_4, 0.05 M KH_2PO_4) using a stomacher (Masticator 400, IUL Instruments, Barcelona, Spain). Next, 100 µl of serial 10-fold dilutions of each extract were spread onto LB agar plates and incubated at 28 ºC for 24 h. *Bacillus*-like colonies were identified on the basis of their morphology [46], by optical microscopy, and by means of PCR directed at the *16S rRNA* genes. Both total bacteria and *Bacillus* population levels were determined.

The presence of *Bacillus* was also determined in the sample extract by PCR using the primers developed for the *16S rRNA* genes. Isolated strains and sample extracts were also analyzed by PCR, using specific primers for the biosynthetic genes *bmyB, fenD, ituC, srfAA*, *bacA*, and *spaS*. The SE method involved thermal treatment of the sample extract at 80 ºC for 10 min followed by enrichment, which consisted of an inoculation (1:100 diluted) in LB for 24 h at 40 ºC. After each of the procedures was completed, the samples were analyzed by PCR using primers directed at the 16S rRNA genes. Only extracts showing amplification for *Bacillus* were further processed for the isolation of *Bacillus* colonies, confirmed as previously described. Finally, the presence of biosynthetic genes (*bmyB, fenD, ituC, srfAA*, *bacA* and *spaS*) was determined both in the extracts and in the *Bacillus* isolates obtained.

The distribution and pattern of AMP biosynthetic genes were examined in 184 *Bacillus* isolates obtained from 183 field samples, including different plant environments (143 samples of the aerial plant part, 25 samples of the rhizosphere, 15 samples of bare soil) and 35 plant species (cultivated, wild-type) collected from seven sampling sites in the Mediterranean area (mainly in Catalonia and the Balearic Islands) of Spain (Table 2). Strain recovery was enhanced by processing the samples using the SE method as previously described. A maximum of three different colonies of the typical *Bacillus* morphology were purified and identified per sample. Finally, the presence of AMP biosynthetic genes in the plant extracts and *Bacillus* isolates was determined by PCR using the specific primers for these genes (*bmyB, fenD, ituC, srfAA*, *bacA*, and *spaS*) as described above.

Table 2. Sampling locations

Location	Latitude	Longitude	Sample type*	Number of samples
Barcelona	41º 21′ 41.63″ N	1º 40′ 09.90″ E	A	7
Girona	42º 03′ 07.25″ N	3º 04′ 00.00″ E	A	12
	42º 03′ 07.03″ N	3º 11′ 46.66″ E	A	23
	42º 14′ 17.39″ N	2º 51′ 25.98″ E	A	4
	42º 02′ 35.44″ N	3º 07′ 34.90″ E	A	3
	42º 03′ 07.03″ N	3º 11′ 46.66″ E	R	13
	42º 02′ 35.44″ N	3º 07′ 34.90″ E	R	4
	42º 03′ 07.03″ N	3º 11′ 46.66″ E	S	4
	42º 02′ 35.44″ N	3º 07′ 34.90″ E	S	6
Lleida	41º 31′ 13.89″ N	0º 52′ 09.13″ E	A	6
	42º 22′ 55.49″ N	1º 06′ 56.29″ E	A	10
	42º 27′ 00.16″ N	1º 13′ 30.68″ E	A	4
	42º 35′ 17.44″ N	1º 19′ 34.89″ E	A	1
	42º 34′ 00.66″ N	0º 55′ 29.10″ E	R	3
	42º 34′ 00.66″ N	0º 55′ 29.10″ E	S	3
	42º 27′ 00.16″ N	1º 13′ 30.68″ E	S	2
Malaga	36º 46′ 43.12″ N	4º 06′ 02.43″ W	A	3
Minorca	40º 03′ 15.39″ N	4º 03′ 15.79″ E	A	8
	40º 05′ 21.91″ N	4º 08′ 31.46″ E	A	11
	39º 59′ 27.22″ N	4º 05′ 37.71″ E	A	10
	40º 05′ 21.91″ N	4º 08′ 31.46″ E	R	4
Navarre	42º 41′ 43.41″ N	1º 40′ 33.85″ W	A	4
Zaragoza	42º 13′ 85.13″ N	1º 53′ 13.60″ W	A	1
	41º 36′ 20.94″ N	1º 14′ 48.12″ W	A	1
	41º 30′ 27.89″ N	1º 24′ 09.12″ W	A	4
Sevilla	37º 20′ 24.41″ N	6º 08′ 19.53″ W	A	2
Tarragona	41º 10′ 19.43″ N	1º 01′ 13.68″ E	A	20
	41º 10′ 19.43″ N	1º 01′ 13.68″ E	R	1
Valencia	38º 54′ 12.53″ N	0º 25′ 02.49″ W	A	8
	39º 11′ 51.65″ N	0º 20′ 03.56″ W	A	1

*A, aerial plant part; R, rhizosphere; S, soil.

Results

Evaluation of PCR assays. PCR using the generalist primers for *Bacillus* directed at the 16S rRNA and *spoVG* genes resulted in amplifications for the four *Bacillus* strains tested. However, primers *spoVG* also amplified three *P. agglomerans* (EPS10, EPS13, and EPS230) and four *P. fluorescens* (EPS173, EPS62e, EPS353, and EPS684) strains at both concentrations tested (10^6 and 10^8 CFU/ml). Primers 16SBACF and 16BACR also amplified three *P. fluorescens* (EPS173, EPS353, and EPS684) strains at 10^8 CFU/ml, although only strain EPS317 was positive at the lowest concentration. The 16S rRNA genes primers were more sensitive than the s*poVG* primers in the four *Bacillus* spp. strains analyzed. Thresholds were established between 5×10^1 and 5×10^4 CFU/ml for the 16S rRNA genes, and between 5×10^3 and 5×10^6 CFU/ml for s*poVG*, depending on the *Bacillus* strain. On the basis of these results, primers for 16S rRNA genes were used for the confirmation of *Bacillus*, but with diluted samples thereof.

For the biosynthetic genes (*bmyB, fenD, ituC, srfAA*, *bacA*, and *spaS*), the sensitivity of primer detection was highly variable, also among the strains. The *fenD* primers were the most sensitive and most consistent among strains, with a detection limit around 10^2 CFU/ml, while *spaS* primers were the least sensitive and least consistent among strains, with a detection limit between 1×10^4 and 1×10^7 CFU/ml. Primers for *srfAA*, *bmyB*, *ituC*, and *bacA* showed a wide threshold of detection, with values between 5×10^2 and 1×10^4 CFU/ml depending on the strain. The specificity study showed that

Table 3. Population levels of total bacteria and *Bacillus* spp. in samples of different plant parts according to two methods of analysis

Method	Origin	Samples	Total culturable bacteria (log_{10} CFU/g)	*Bacillus*-like (log_{10} CFU/g)	Positive samples for *Bacillus*		Confirmed *Bacillus*	
					16S rRNA genes	≥ 3 AMP*	Isolates number	≥ 3 AMP*
Standard	Aerial	15	6.01 ± 1.59	0.29 ± 1.11	14	8	2	2
	Rhizosphere	15	7.08 ± 0.65	2.54 ± 2.88	5	2	9	4
	Soil	15	6.62 ± 0.75	2.80 ± 2.75	3	1	7	7
	Total	45	6.57 ± 1.00	1.88 ± 2.60	22	11	18	13
Enrichment	Aerial	15	8.10 ± 2.63	6.60 ± 3.32	15	13	14	13
	Rhizosphere	15	8.03 ± 1.49	7.55 ± 2.50	15	14	19	11
	Soil	15	7.29 ± 0.78	6.41 ± 2.31	15	10	22	17
	Total	45	7.81 ± 1.80	6.85 ± 2.73	45	37	55	41

*Three or more antimicrobial peptide genes simultaneously.

genes related to the biosynthesis of AMPs were differently distributed among the *Bacillus* strains used as reference. *srfAA* was detected in all 14 strains, except RGAF84, while the other genes were very rare; *spaS* was detected only in UMA6614 and EPS2004, and *ituC* only in three strains. The distribution pattern of the six AMP genes among these 14 *Bacillus* strains was strain-dependent. However, none of the AMP genes was found in RGAF84. In the remaining strains, the distribution patterns were as follows: *srfAA* (RGAF5); *srfAA* and *bacA* (RGAF9, RGAF11, RGAF101); *srfAA, bacA* and *bmyB* (RGAF66, RGAF32, RGAF46), *srfAA, bacA, bmyB* and *fenD* (FZB42); *srfAA, bacA, bmyB, fenD*, and *ituC* (QST713, RGAF51, UMAF6639); *srfAA, bacA, bmyB, fenD* and *spaS* (UMAF6614, EPS2004) were identified. None of the strains had all six AMP genes.

Generally, unspecific amplifications of the AMPs genes were not observed in the strains of the other bacterial species, except for *P. fluorescens* (EPS282 and EPS353), which amplified for *bmyB* primers at a concentration of 10^6 CFU/ml.

Validation of the isolation procedure. In the validation experiment, the population of total culturable bacteria ranged from 6.01 to 7.08 log_{10} CFU/ml, whereas the population of *Bacillus* ranged from 0.29 to 2.8 log_{10} CFU/g (Table 3). A low presence of *Bacillus* was determined in the field samples, although the proportion of *Bacillus* spp. in the total population was higher in rhizosphere and soil samples than in the aerial plant part. In addition, the frequency distribution of the population of total bacteria and of *Bacillus* spp. did not follow a normal distribution (according to the Shapiro-Wilk normality test, $P < 0.05$ in both cases). PCR using 16S rRNA genes detected *Bacillus* spp. in 22 out of 45 samples and in 18 putative *Bacillus* colonies that were isolated (2 from aerial plant parts, 9 from rhizosphere, and 7 from soil). Following the application of the SE method, *Bacillus* population levels increased until they reached high concentrations, with a consistent presence among the different sample types. Total culturable bacteria population levels were 7.29–8.10 log_{10} CFU/g compared to *Bacillus* spp. population levels of 6.41–7.55 log_{10} CFU/g. With the SE method, 55 putative *Bacillus* colonies were isolated, 14 from aerial plant samples, 19 from rizhosphere, and 22 from soil. After enrichment the 16S rRNA gene primers were amplified in all samples, indicating the presence of *Bacillus* spp. in all cases. Thus, the yield obtained by the SE method was around threefold that obtained with the ST method. Furthermore, 21.9 % of *Bacillus* spp. isolates came from aerial plant part samples, while 38.3 % and 39.7 % of isolates came from rhizosphere and soil samples, respectively.

The presence of the six AMP genes (*bmyB, fenD, ituC, srfAA, bacA*, and *spaS*) was determined in natural extracts and in the corresponding *Bacillus* isolates. The methods differed in the frequency of AMP genes per sample. In the ST method, 11 out of 45 samples contained three or more AMP genes whereas in the SE method this was the case in 37 out

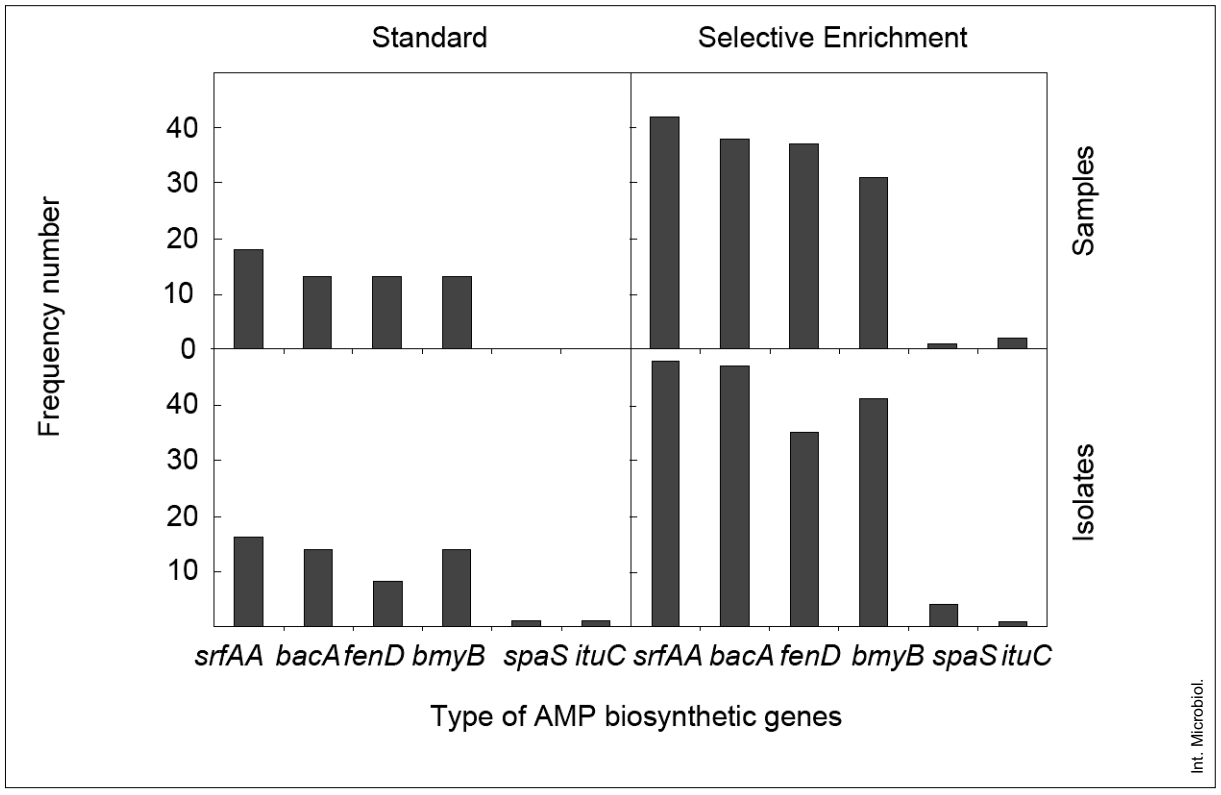

Fig. 1. Type of antimicrobial peptide biosynthetic genes in field samples and in the corresponding *Bacillus* isolates processed directly by the standard method or the selective enrichment method.

of 45 samples (see also Table 3). There were significant differences between the ST and SE methods in terms of the frequency of distribution of the number of genes per sample (c^2, $P < 0.0001$), although the differences were not significant ($P = 0.054$) in the *Bacillus* isolates. Figure 1 shows the distribution frequencies of the six AMP genes for the samples and the *Bacillus* isolates depending on the method used (ST, SE). In field samples and isolates, the frequency distributions were similar (c^2, $P = 0.900$) and independent of the method used. Therefore, the SE method was used to build-up a larger collection of isolates, composed of 184 *Bacillus* strains, submitted to AMP gene analysis using the primer sets previously developed.

Patterns of AMP genes in *Bacillus* strains. The distribution of the number of genes per strain and the frequency of each gene within the population are shown in Fig. 2. The number of simultaneous genes per strain followed a normal distribution (Fig. 2A). Most isolates had at least one of the biosynthetic genes (171 isolates, 92.9 %) and the majority of strains had 2–4 simultaneous genes (76.1 %). The simultaneous presence of five genes was rare (3.3 % strains); none of the isolates had all six genes. The distributions of the number of genes were similar among aerial and rhizosphere samples but differed with soil isolates, in which the numbers of genes tended to be higher (3–4). The most frequent genes were *srfAA* (69.0 %), *bacA* (61.4 %), and *bmyB* (55.4 %), followed by *fenD* (40.2 %). The genes *ituC* (9.8 %) and *spaS* (15.2 %) were less frequent (Fig. 2B). These frequencies did not differ significantly by sample origin ($P > 0.05$). Figure 3 shows the patterns of AMP gene markers and the frequencies of these genes among strains. The most frequent genotypes were *srfAA-bacA-bmyB* (15.2 %) and *srfAA-bacA-bmyB fenD* (14.1 %). Isolates containing at least *srfAA, srfAA-bacA*, or *srfAA-bacA-bmyB* accounted for 69 %, 46.7 %, and 34.8 %, respectively.

Discussion

Studies on AMP biosynthetic genes in natural populations of *Bacillus* may be useful for discovering new inoculant strains with broad-ranging and better efficacy of pathogen control as well as improved fitness in plant environments. The

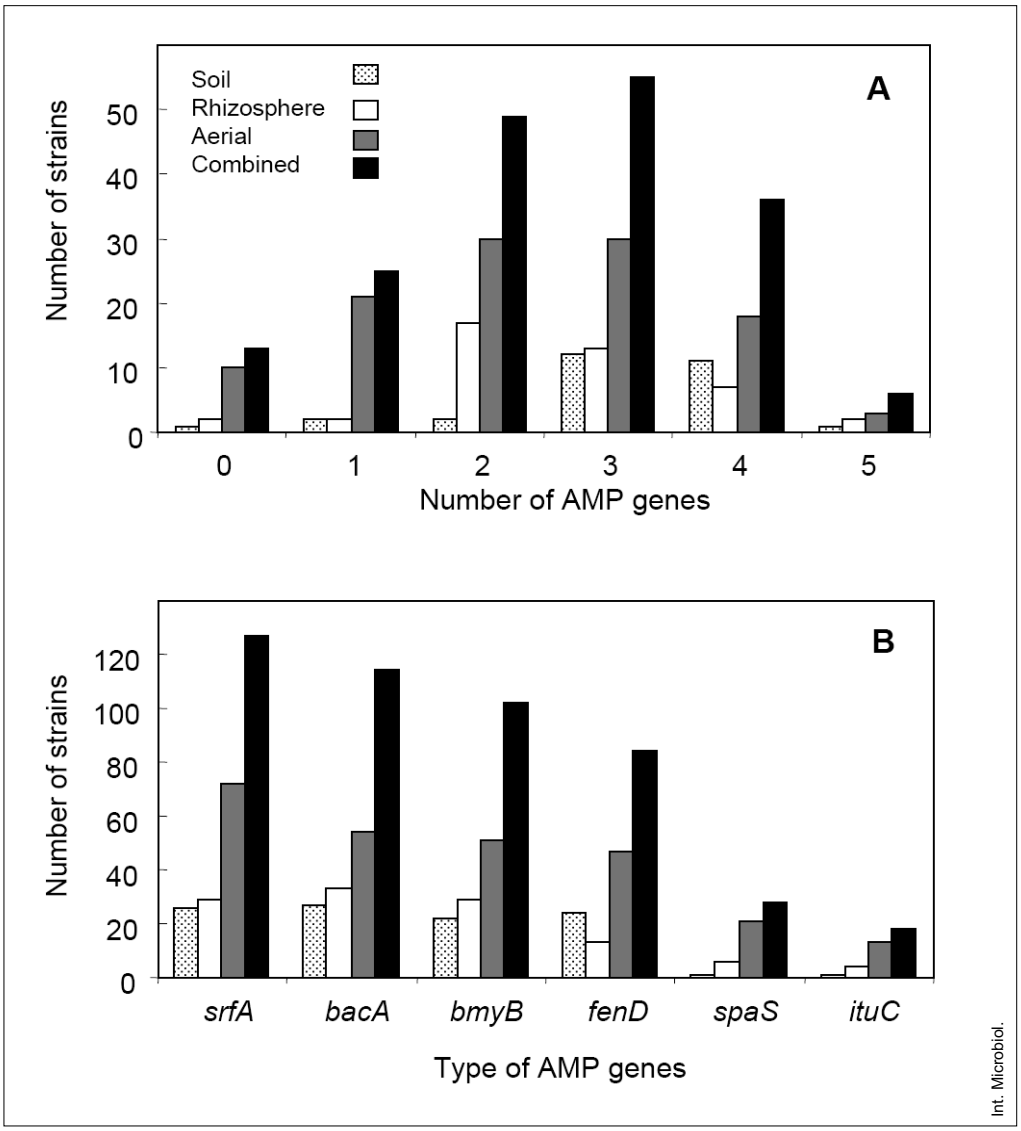

Fig. 2. Frequency distribution of the number (**A**) and type (**B**) of antimicrobial peptide biosynthetic genes in 184 strains of *Bacillus* spp. isolated from field samples. Data are presented separately by sample origin (soil, rhizosphere, aerial plant part or combined).

sequenced genomes of *B. amyloliquefaciens* FZB42 and *B. subtilis* 168 together with the finding of relevant genes in other strains has been applied to the identification of functional molecular markers related to secondary metabolite production (e.g. AMPs) and to their beneficial effects in plants [10]. In turn, this has facilitated the development of PCR tools to analyze the prevalence and distribution of these markers in commercial biocontrol *Bacillus* strains and in isolates from natural populations.

In the present study, we developed sensitive PCR methods to analyze six AMP genes reportedly related to the antagonistic capacity of *Bacillus* [23]. The study of these markers in a reference collection of *Bacillus* spp. confirmed the presence of *srfAA*, *bacA*, *bmyB*, and *fenD* in strain FZB42, and of *srfAA*, *bmyB*, *fenD*, and *ituC* genes in strain QST713, in agreement with previous reports [23,26]. However, we detected a fifth AMP gene, coding for *bacA*, in strain QST713. Although these AMP gene markers have not been reported previously in other strains, our results are consistent with the production of bacillomycin, fengycin, and surfactin detected by other authors in strain UMAF6614 and of iturin, fengycin and surfactin in strain UMAF6639 [46]. Note that we detected gene markers for bacilysin in both strains, for bacillomycin in UMAF6639, and for subtilin in UMAF6614.

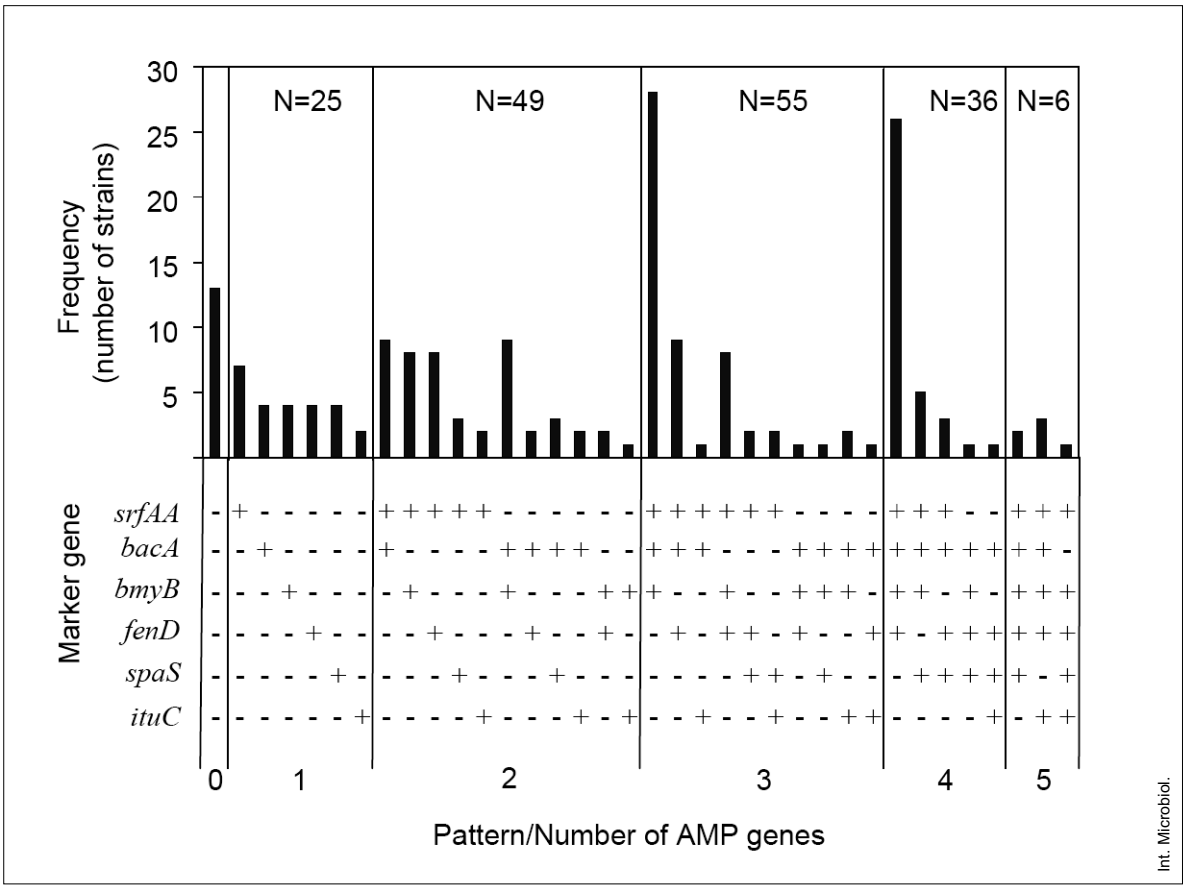

Fig. 3. Frequency distribution of patterns of antimicrobial peptide biosynthetic gene markers in 184 strains of *Bacillus* spp. isolated from field samples. The number of strains (N) within each group of simultaneous number of gene markers is indicated in the upper part of the panels. The presence (+) or absence (–) of antimicrobial peptide biosynthetic gene markers is also indicated.

The *Bacillus* population levels in samples from plants obtained for the present work were frequently very low (< 2.8 $\log_{10}$ CFU/g), in accordance with other reports in which they were either not detected or varied strongly with sample type and origin, with isolates being more frequently obtained from soil or rhizosphere than from aerial plant parts [1,21,33]. Also, the primers we developed for the AMP markers were sensitive enough in some cases but not in others. Consequently, a method to increase *Bacillus* population levels in the extracts from natural samples was developed and validated. This SE method consisted of thermal treatment at 80 ºC for 10 min followed by a cultivation stage at 40 ºC for 24 h. The method was validated for its capacity to recover *Bacillus* populations in complex communities, similar to other studies in which enrichment procedures have been used to increase the yield of isolation of *Bacillus thuringiensis* for insect pest control [7]. A comparison of the SE and ST methods in terms of *Bacillus* isolation yield and the distribution of AMP genes within isolates showed that higher yields were obtained with the former, whereas the two methods did not significantly differ regarding the frequency distribution of genes, thus providing evidence that the SE method did not distort the actual *Bacillus* population structure in the original sample.

Screening of the collection of isolates obtained from field samples covering a wide range of plant environments (soil, rhizosphere, and aerial plant parts) within a Mediterranean land area in Spain indicated a prevalence of AMP gene markers within the *Bacillus* population, since most strains had at least one marker. Note that the number of gene markers per strain followed a normal distribution, with the most frequent values being 2–4. The dominant gene markers were *srfAA*, *bmyB*, *bacA*, and *fenD*, with *ituC* and *spaS* only scarcely represented. This high presence of certain AMP gene markers among the *Bacillus* population is in agreement with the fact that the genomes of strains *B. subtilis* 168 and *Bacillus amy-*

loliquefaciens FZB42 have six AMP operons [26]. However, in a study of the gene markers of bacillomycin D, iturin A, surfactin, mycosubtilin, fengycin, and zwittermicin A within a collection of strains antagonistic to *S. sclerotiorum*, the majority of strains were found to harbor sufactin and iturin [5]. Our results are in agreement with the prevalence of the surfactin gene, but not in the case of iturin gene. This may be due to the fact that in the above report the strain collection included only strains active against the target pathogen, whereas in our study the strains were not selected for a specific pathogen. In addition, those authors analyzed *ituA* rather than *ituC*.

The finding that *srfAA*, *bmyB*, *bacA*, and *fenD* genes are dominant in plant environments reinforces the competitive role of surfactin, bacyllomicin, fengycin, and bacilysin in conferring strain fitness in natural environments. Surfactin production is widespread among *B. subtilis* and *B. amyloliquefaciens* and it has been implicated both in cell attachment and detachment to surfaces during biofilm formation and in swarming motility [39,42]. For example, the colonization of plant roots by *B. subtilis* is associated with surfactin production and biofilm formation. In addition, surfactin has been linked to the protection of *Arabidopsis thaliana* against infection by the pathogen *P. syringae* pv. tomato [6]. Bacyllomicin, a member of the iturin family, has been reported together with fengycin to have strong antifungal activity [52], being responsible for the main antagonistic activity of *B. amyloliquefaciens* FZB42 against *Fusarium oxysporum* [27]. In the case of bacilysin, activity against a wide range of bacteria has been reported [11,30].

The fact that the most abundant pattern observed in our isolates was *srfAA-bmyB-bacA*, either alone or with *fenD*, suggests a certain degree of linkage between these genes. The probability of paired associations of these gene markers is not related to their relative distance, at least on the basis of genome sequences and relative distances in known strains of *Bacillus* [10,26]. Alternatively, horizontal genetic exchange followed by a positive selection pressure within the population can be postulated. In fact, the uptake of phage, plasmid, or naked DNA, including clustered non-ribosomal protein synthesis (NRPSs) genes, has been suggested for genetically competent cells of *Bacillus* strains 168, FZB42, and GA1 due to the presence of insertion sequence elements [3,15]. In addition, the predominance of the above-mentioned four genes in the *Bacillus* population could be due to the benefit provided by complementary mechanisms of action among the gene products, including the biosurfactant and biofilm activating properties of surfactin, the antifungal activity of bacillomycin and fengycin, and the antibacterial activity of bacilysin.

While we have demonstrated the prevalence of several AMP genes in plant-associated populations of *Bacillus*, the relationship of the presence of these genes with the antagonistic capacity of the respective strains and with the synthesis of the expression products remains to be determined. The identification of these gene markers in *Bacillus* strains can be applied to increase the yield of putative biological control agents for a wide range of plant pathogens.

Acknowledgements. This work has been supported in part by project AGL2009-13255-C02-01 from Spanish Ministry of Science and Innovation. The research group has been accredited by the CIRIT of the Autonomous Government of Catalonia (2009SGR812).

Competing interests: None declared

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