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A mixed-species microarray for identification of food spoilage bacilli

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ABSTRACT

Failure of food preservation is frequently caused by thermostable spores of members of the Bacillaceae family, which show a wide spectrum of resistance to cleaning and preservation treatments. We constructed and validated a mixed-species genotyping array for 6 *Bacillus* species, including *Bacillus subtilis, Bacillus licheniformis, Bacillus pumilus, Bacillus sporothermodurans, Bacillus cereus and Bacillus coagulans*, and 4 *Geobacillus* species, including *Geobacillus stearothermophilus, Geobacillus thermocatenulatus, Geobacillus toebii and Geobacillus* sp., in order to track food spoilage isolates from ingredient to product. The discriminating power of the array was evaluated with sets of 42 reference and 20 test strains. Bacterial isolates contain a within-species-conserved core genome comprising 68–88% of the entire genome and a non-conserved accessory genome comprising 7–22%. The majority of the core genome markers do not hybridise between species, thus they allow for efficient discrimination at the species level. The accessory genome array markers provide high-resolution discrimination at the level of individual isolates from a single species. In conclusion, the reported mixed-species microarray contains discriminating markers that allow rapid and cost-effective typing of *Bacillus* food spoilage bacteria in a wide variety of food products.

1. Introduction

Outgrowth of aerobic spore-forming bacteria in food products is a frequent cause of spoilage as a result of the extreme resistance of their spores to heat and other preservation treatments (Kort et al., 2005; Setlow, 2006). The majority of these spores originate from the mesophilic genus *Bacillus* and the thermophilic genus *Geobacillus* and occur in a wide variety of processing environments (Brul et al., 2006; Oomes et al., 2007; Scheldeman et al., 2005). The proposed genus *Geobacillus* contains thermophilic species previously classified in the genus *Bacillus* (Nazina et al., 2001). Both the *Geobacillus* and *Bacillus* genera belong to Bacillaceae, a family of Gram-positive, heterotrophic, rod-shaped bacteria of which all members produce endospores (De Vos et al., 2008). Most members of the *Bacillaceae* are non-pathogenic, but some *Bacillus* species are known to cause disease in humans, like *Bacillus anthracis* causing anthrax (Madigan and Martinko, 2005) and certain *Bacillus cereus* strains causing foodborne illnesses via the production of diarrhoeal or emetic enterotoxins (Kotiranta et al., 2000).

Since tracking and tracing of closely related bacterial isolates, often with diverse phenotypes (Earl et al., 2008), is laborious and technically demanding (Oomes et al., 2007), we set out to develop a rapid identification system for pure bacterial isolates, based on genomic variability, which is known to be relatively high in *Bacillus* (Seki et al., 1975; Earl et al., 2007). We have constructed a microarray containing genetic information of 34 different strains from *Bacillus* and *Geobacillus* genera allowing for high-resolution genotyping by comparative genome hybridisations. We show that this microarray-based comparative genome hybridisation (M-CGH) is a powerful tool for the rapid and unambiguous discrimination of genetically distant as well as closely related isolates of spore-forming bacilli in the food chain.

2. Materials and methods

2.1. Array construction and hybridisation

The microarray was essentially constructed, hybridised, washed and scanned as described previously (Vlaminckx et al., 2007). Briefly, genomic microbial DNA was isolated from 34 reference food spoilage strains (Table 1A). Equimolar amounts of DNA were mixed into 7 pools each containing 4 to 9 strains: 6 pools of *Bacillus*

Abbreviations: CGH, comparitive genome hybridisation; M-CGH, microarray-based comparative genome hybridisation.

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Table 1
A and B summarize details of reference and test strains respectively.

A Reference strains						
Nr[a]	TTC[b]	16S Ribo[c]	Strain[d]	Ref[e]	Sampled from[f]	M-CGH type[g]
1	02.0196	*B. su*	168 PS832	2	–	*B. su* 1/(2/3)
2	02.0197	*B. su*	168 1A1	1, 2	–	*B. su* 2/3/(1)
3	03.0138	*B. su*	168 1A700	2	–	*B. su* 2/3/(1)
4	02.0195	*B. su*	A163	3,7	Peanut chicken soup	*B. su* 4
5	02.0199	*B. su*	CC16	4,7	Curry cream soup	*B. su* 5
6	02.0194	*B. su*	CC2	4,7	Curry cream soup	*B. su* 6
7	02.0200	*B. su*	IIC14	4,7	Binding flour	*B. su* 7/(9)
8	02.0198	*B. su*	MC85	4,7	Curry soup	*B. su* 8
9	02.0201	*B. su*	RL45	4,7	Red lasagna	*B. su* 9/(7)
10	03.0143	*B. li*	E5	4,7	Pea soup	*B. li* 10
11	00.0156	*B. li*	LMG 6933	5	–	*B. li* 11
12	03.0154	*B. li*	T1	4,7	Pea soup	*B. li* 12
13	03.0155	*B. li*	T29	4,7	Mushroom soup	*B. li* 13
14	02.0051	*B. pu*	TNO isolate	6	–	*B. pu* 14
15	02.0142	*B. pu*	TNO isolate	6	–	*B. pu* 15
16	03.0141	*B. pu*	R1	4,7	Reis-eintopf (stew)	*B. pu* 16
17	03.0142	*B. pu*	R2	4,7	Reis-eintopf (stew)	*B. pu* 17
18	02.0203	*B. sp*	IC14	4,7	Indian curry	*B. sp* 18
19	02.0202	*B. sp*	IC4	3,7	Indian curry	*B. sp* 19
20	02.0204	*B. sp*	IIC65	4,7	Black fungus	*B. sp* 20
21	03.0166	*B. ce*	-	6	–	*B. ce* 21
22	02.0043	*B. ce*	-	6	–	*B. ce* 22
23	03.0152	*B. ce*	LMG 6923	5	–	*B. ce* 23
24	01.0142	*B. ce*	-	6	–	*B. ce* 24
25	02.0205	*B. co*	IC1	4,7	Indian curry	*B. co* 25
26	03.0139	*B. co*	C1	4,7	Chinese tomato	*B. co* 26
27	03.0140	*B. co*	C2	4,7	Chinese tomato	*B. co* 27
28	03.0156	*B. co*	PS6	4,7	Tomato supreme	*B. co* 28
29	03.0157	*G. st*	T4	4,7	Pea soup	*G. st* 29
30	03.0159	*G. st*	T26	4,7	Pea soup	*G. st* 30/(34)
31	03.0160	*G. th*	T9	4,7	Pea soup	*G. th* 31
32	03.0161	*G. —*	T22	4,7	Mushroom soup	*G. (th)*32
33	03.0162	*G. to*	T27	4,7	Pea soup	*G. to* 33
34	03.0158	*G. st*	T14	4,7	Pea soup	*G. st* 34/(30)
35	03.0101	*S. au*	UMCU 01A254	6	Clinical Isolate	non *B.*
36	03.0117	*S. au*	UMCU 10A172	6	Clinical Isolate	non *B.*
37	04.0072	*E. cl*	UMCU 02.406	6	Clinical Isolate	non *B.*
38	04.0082	*E. cl*	UMCU 03.089	6	Clinical Isolate	non *B.*
39	04.0291	*S. py*	UMCU 9600284	6	Clinical Isolate	non *B.*
40	04.0294	*S. py*	UMCU 9600656	6	Clinical Isolate	non *B.*
41	04.0236	*E. fa*	UMCU E0155	6	Clinical Isolate	non *B.*
42	04.0250	*E. fa*	UMCU E1284	6	Clinical Isolate	non *B.*
B Test strains						
Nr[a]	TTC[b]	16S Ribo[c]	Ref. Id[d]	Ref[e]	Sampled from[f]	M-CGH type[g]
43	05.0458	*B. su*	A162	7	Peanut chicken soup	*B. su* 43
44	04.0319	*B. —*	–	7	Oyster sauce	*B. su* 44/45
45	04.0320	*B. —*	–	7	Oyster sauce	*B. su* 44/45
46	04.0321	*B. —*	–	7	Oyster sauce	*B. su* 46
47	04.0322	*B. —*	–	7	Kerry powder	*B. su* 47
48	04.0327	*B. —*	–	7	Sweet pepper powder	*B. su* 48
49	04.0331	*B. —*	–	7	Oyster sauce	*B. su* 49
50	04.0324	*B. li*	–	7	Kerry powder	*B. li* 50
51	04.0325	*B. —*	–	7	Kerry powder	*B. li* 13
52	04.0312	*B. pu*	–	7	Coriander powder	*B. pu* 52/55
53	04.0314	*B. pu*	–	7	Lemon peal	*B. pu* 53
54	04.0323	*B. pu*	–	7	Kerry powder	*B. pu* 14
55	04.0328	*B. pu*	–	7	Sweet pepper powder	*B. pu* 52/55
56	05.0459	*B. —*	PS3	7	Chinese tomato soup	*B. co* 56
57	05.0460	*B. —*	PS4	7	Chinese tomato soup	*B. co* 57
58	05.0461	*B. —*	PS5	7	Chinese tomato soup	*B. co* 58
B Test strains						
Nr[a]	TTC[b]	16S Ribo[c]	Ref. Id[d]	Ref[e]	Sampled from[f]	M-CGH type[g]
59	05.0168	*G. st*	T21	7	Mushroom soup	*G. st* 59
60	05.0158	*G. —*	T7	7	Pea soup	*G. to* 60
61	05.0170	*G. st*	T23	7	Mushroom soup	*G. st* 61
62	05.0185	*G. st*	T42	7	Chicken soup	*G. st* 30/34

[a] Sequential strain numbers allowing identification in other figures and tables.
[b] TNO Type Strain Collection Id's (TTC).
[c] Putative identification by 16S-rDNA sequencing or Ribotyping (*B. su* = *Bacillus subtilis, B. li* = *B. licheniformis, B. pu* = *B. pumilus, B. sp* = *B. sporothermodurans, B. ce* = *B. cereus, B. co* = *B. coagulans, G. st* = *Geobacillus stearothermophilus, G. th* = *G. thermocatenulatus, G. to* = *G. toebii, G.—* = G. sp., *S. au* = *Staphylococcus aureus, E. cl* = *Enterobacter cloacae, S. py* = *Streptococcus pyogenes and E. fa* = *Enterococcus faecium*).
[d] Strain/Isolate Id. from reference[e].
[e] Reference paper or lab: 1 = Burkholder and Giles (1947), 2 = *Bacillus* Genetic Stock Centre (www.bgsc.org), 3 = Kort et al., 2005, 4 = Oomes et al., 2007, 5 = BCCM/LMG Bacteria Collection (bccm.belspo.be), 6 = TNO Type Strain Collection (author), 7 = Unilever Strain Collection (author).
[f] Food matrix from which strain was isolated.
[g] *Bacillus* species type determined via M-CGH, numbers indicate specific isolates based on unique M-GCH patterns (Fig. 3).

species and 1 pool *Geobacillus* genus (Table 2). DNA mixtures of each of the 7 pools were sheared by mild sonication. DNA fragments of 1–2 kb were isolated from agarose gels, end repaired (Lucigen, Middleton, USA), cloned into pSmartHCkan vectors (Lucigen, Middleton, USA) and transformed to *Escherichia coli*, resulting in 7 libraries Inserts were PCR amplified from randomly picked *E. coli* transformants and spotted on 5760-feature glass-arrays, containing representatives of all 7 Bacillaceae gene libraries (Table 2). Genomic DNA of 8 non-*Bacillus* strains (outgroup), the 34 reference strains (Table 1A) and 20 test strains (Table 1B) was labelled with Cy5-dUTP and hybridised on the arrays at 42 °C in Easy Hyb buffer (Roche). Several reference strains 1–42 (Table 1A) were hybridised in replicate (Fig. 1A). After laser scanning (ScanArray 5000, PerkinElmer Life Sciences), the Cy5-fluorescence images were converted into median signal (S) and median background (B) values for each spotted genomic DNA fragment, further referred to as marker.

A set of array markers showing confirmative differential hybridisation (Fig. 3) were identified by bidirectional sequencing (read length 500–1000 bp, using pSmart primers SR2 and SL1) followed by protein homology searches in the Swissprot database at NCBI using the BLASTX algorithm (http://www.ncbi.nlm.nih.gov/).

2.2. Data processing and analysis

The raw data were processed using Microsoft Office Excel 2002 Service Pack 2 as described previously (Vlaminckx et al., 2007). Briefly, the array signal (S) to background (B) ratios (S/B) were normalised based on the average S/B ratio (avg(S/B)) for all markers per array with S/B larger than 40 and S smaller than 62 000 (n = Avg (S/B) per array). Next, the overall normalisation factor N for all the arrays in the study was calculated (N = Avg(n)), and used for

Table 2
Reference *Bacillus* strains represented on the mixed-species array.

Genomic libraries (Id. and species):	L1 *B.su*	L2 *B.li*	L3 *B.pu*	L4 *B.sp*	L5 *B.ce*	L6 *B.co*	L7: *G.* *st*	*th*	*to*	—	Total
Nr of Ref. Strains:	9	4	4	3	4	4	3	1	1	1	34
Nr of markers:	2304	768	192	768	192	768			768		5760

Nr. of mixed reference strains and genomic markers per species-library (L1–7) are given. For abbreviations see Table 1.