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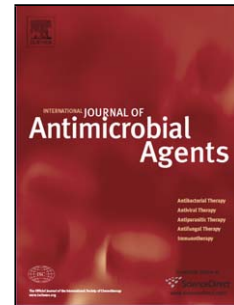
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Study on the dissemination of the *bcrABDR* cluster in *Enterococcus* spp. reveals that the BcrAB transporter is sufficient to confer high-level bacitracin resistance

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Abstract

Bacitracin is used both in clinical practice and as an animal growth promoter in some countries. Recent reports revealed the dissemination of high-level bacitracin resistance (HLBR) in the genus *Enterococcus*. The *bcrABD* operon confers HLBR in an *Enterococcus faecalis* strain, and the presence of the regulator BcrR was stated as essential for this phenotype. Our objectives were to investigate the spread of the *bcrABDR* genes in the genus *Enterococcus* and to evaluate the role of this genotype in the HLBR phenotype. Fifty-eight enterococcal isolates from different environments and species were screened for the presence of the *bcrABDR* genes, and minimum inhibitory concentrations for bacitracin were determined. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to study the expression of bacitracin resistance genes in strains with HLBR following growth in the presence and absence of the antibiotic. The *bcrABDR* genotype was found associated with *E. faecalis* strains from clinical settings bearing a HLBR phenotype. The most disseminated mechanism of bacitracin resistance was the BcrAB ABC transporter, encoded by the *bcrAB* genes. This transporter, which is constitutively expressed in the absence of *bcrR*, was sufficient to confer HLBR. Our findings stress the need for further evaluation of bacitracin resistance in this genus.

1. Introduction

Enterococci inhabit the gastrointestinal tract of humans and other mammals, birds and insects. They are also found in other environments, such as food, sand and water. For many years these microorganisms have been considered harmless to man. However, this vision has been changing in recent decades. *Enterococcus* spp. are now the second cause of surgical and urinary tract infections and the third cause of bacteraemia [1]. Antibiotic use as growth promoters in animal feed, together with its application in clinical therapy, are two of the main factors responsible for the increase in infections caused by multiresistant enterococci [2].

Between 1997 and 1999 some growth-promoting antibiotics, namely avoparcin, bacitracin, spiramycin, tylosin and virginiamycin, were withdrawn as growth promoters in the European Union on the basis of the Precautionary Principle [3]. Although banned in Europe as a growth promoter since 1999, bacitracin is still used for this purpose in other countries, namely New Zealand [4]. This potent antibiotic is currently used in some topical preparations both in human and veterinary medicine and has also been tested, with limited success, for elimination of vancomycin-resistant enterococci [5]. Bacitracin is not administered systemically owing to its nephrotoxicity but is considered safe when taken orally as the gastrointestinal tract does not absorb significant amounts of the drug [6,7].

Almost one decade after withdrawal of bacitracin from animal feeding in Europe, some reports appeared recently revealing relevant phenotypic detection of bacitracin

resistance [8–10]. This polypeptide antibiotic is produced by *Bacillus licheniformis* and some strains of *Bacillus subtilis* and acts as an inhibitor of cell wall synthesis, although it may also interfere with other cellular processes [11]. Bacitracin forms a complex, mediated by a metal ion, with lipid undecaprenyl pyrophosphate (UPP), a carrier of the *N*-acetylmuramyl pentapeptide intermediates for the synthesis of peptidoglycan. By binding to UPP, bacitracin prevents its dephosphorylation and consequently the recycling of the carrier, thus inhibiting cell wall synthesis. Two main resistance mechanisms have been described in Gram-negative and Gram-positive bacteria. The *bacA* gene of *Escherichia coli* was the first to be identified, in 1992. BacA has recently been renamed as UppP, an undecaprenyl pyrophosphate phosphatase, which generates undecaprenol monophosphate (UP) from UPP, thus contributing to resistance to bacitracin [10]. BacA homologues have also been identified in other bacteria, namely *Streptococcus pneumoniae*, *Staphylococcus aureus* and the *Enterococcus faecalis* V583 genome [12]. A second resistance mechanism, the BcrABC transporter that pumps out bacitracin, was identified in 1995 in *B. licheniformis* [13]. Homologues of this transporter have been identified in other bacteria, namely *B. subtilis*, *Streptococcus mutans* and *E. faecalis* [14]. A third mechanism of resistance, reported in *Xanthomonas campestris*, *Sphingomonas* sp., *E. coli* and *S. mutans*, relies on exopolysaccharide production, which diminishes the entry of bacitracin into the cell [15].

In 2004, Manson et al. [16] reported an ABC transporter system involved in bacitracin resistance in *E. faecalis*, in addition to an overproduced undecaprenol kinase. This enzyme converts UPP to UP, increasing the amount of lipid carrier present in the cell, thus overcoming the sequestration of UPP by bacitracin and increasing the resistance of

the organism to the antibiotic [11]. These two mechanisms were both encoded by the *bcrABD* operon, which in turn was under the control of a regulatory gene, *bcrR* (see Fig. 3) [16]. The presence of this gene cluster conferred high-level bacitracin resistance (HLBR), defined as a minimum inhibitory concentration (MIC) >256 mg/L. BcrR has been identified as both a membrane-bound sensor and transducer of bacitracin availability to regulate *bcrABD* expression and produce HLBR [17].

Despite this recent discovery, studies on enterococcal bacitracin resistance mechanisms remain limited and breakpoints between susceptibility and resistance remain to be set. In fact, no Clinical and Laboratory Standards Institute breakpoints exist for bacitracin, and authors appear to adopt different criteria to define bacitracin-resistant enterococci [4,7]. Moreover, no clear correlation between phenotype, i.e. MIC, and genotype has ever been established.

Considering that bacitracin usage has an impact in several environments, from agriculture to medicine, enterococcal isolates from different environments and species were screened for resistance to this antibiotic. MICs were determined and the presence of *bcrABDR* genes was assessed by polymerase chain reaction (PCR). Some strains with HLBR did not carry the *bcrR* gene and this was further investigated. Two strains with HLBR carrying *bcr* genes but differing in the presence of *bcrR* were grown in the presence and absence of bacitracin. Growth curves and reverse transcriptase (RT)-PCR indicated that in the absence of the *bcrR* gene, expression of *bcrABD* genes is constitutive, thus demonstrating that BcrR is not required for HLBR in *E. faecalis*. To our

knowledge, this is the first time a large collection of enterococcal isolates has been screened for the molecular mechanisms underlying bacitracin resistance in this genus.

2. Materials and methods

2.1. Microorganisms

A total of 58 enterococcal isolates from different environments were used in this study: 17 strains from dairy products (cheese and milk), 10 from water (both drinking water and from lakes), 14 from sand and 17 from human (hospital) and animal infections. All isolates were previously identified as described by Alves et al. [18] and D. Pinto et al. (personal communication).

2.2. Minimum inhibitory concentration determination

MICs for bacitracin were determined by Etest (AB BIODISK, Solna, Sweden) according to the manufacturer's instructions. Each plate was inoculated with a suspension of the microorganism equivalent to 0.5 McFarland turbidity standard [ca. 1.5×10^8 colony-forming units (CFU)/mL].

2.3. DNA preparation

Total DNA was extracted from cells according to the method of Serror et al. [19].

2.4. Polymerase chain reaction

PCR was used to screen for *bcrA*, *bcrB*, *bcrD* and *bcrR* genes and was performed on a TPpersonal combi thermocycler (Biometra, Göttingen, Germany). Each 20 μ L of PCR reaction mixture contained 1 \times Eppendorf MasterMix (Eppendorf, Madrid, Spain), 1 μ M of each primer (Table 1), 100 ng of DNA and water. Primers were purchased from MWG-Biotech (Ebersberg, Germany). The thermocycler was programmed with the following conditions: 5 min at 95 $^{\circ}$ C; 30 s at 95 $^{\circ}$ C, 45 s at 51 $^{\circ}$ C and 30 s at 72 $^{\circ}$ C for 30 cycles; 5 min at 72 $^{\circ}$ C; and 4 $^{\circ}$ C until analysis. Visualisation of amplicons was done with ethidium bromide (Sigma, Hamburg, Germany) under ultraviolet irradiation after electrophoresis on a 1% agarose gel. Image analysis was performed with Kodak Digital software.

2.5. Statistical analysis

Homogeneity tests were conducted with adjustment to χ^2 test in an attempt to associate the presence of *bcrABDR* genes with bacitracin MICs.

2.6. Reverse transcriptase polymerase chain reaction

Two strains with HLBR (*E. faecalis* 1213 and *Enterococcus hirae* LN85) were grown in the absence and presence of 64 mg/L bacitracin, corresponding to 0.25 \times their MIC values. Growth was performed in brain–heart infusion broth inoculated with 1% inoculum and followed by measuring the optical density (OD) at 600 nm. Cells were harvested at two key points of the growth curve: OD 0.2, corresponding to early

exponential phase; and OD 0.8, corresponding to late exponential phase. Growth experiments were performed in triplicate. RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Expression of *bcrAB* genes was assessed by RT-PCR. Production of cDNA was performed with a SuperScript™ First-Strand Synthesis System (Invitrogen, Barcelona, Spain) according to the manufacturer's instructions. PCR was performed with 2 µL of cDNA using primers 3 and 1 (Table 1). The expected amplicon size was 1503 bp.

2.7. Sequencing of the *bcrAB* promoter region

Primers 2 and 7 (Table 1) were used to amplify the intergenic region between the *bcrR* and *bcrA* genes of strains *E. hirae* LN85 (carrying the genotype *bcrABR*) and *E. faecalis* B23897 (carrying the *bcrABDR* genotype). Sequencing of the upstream region of the *bcrA* gene in strain *E. faecalis* 1213 (carrying the *bcrAB* genotype) was performed using primers 9 and 10 (Table 1). Primer 9 was targeted at the transposase gene [16]. PCR products were purified using QIAquick® PCR Purification Kit (Qiagen) according to the manufacturer's instructions and sent to BaseClear (Leiden, The Netherlands) for sequencing.

3. Results and discussion

The peptide antibiotic bacitracin is not heavily used either in the hospital setting or in agriculture and was banned from animal feeding in Europe almost 10 years ago. However, recent work reported high levels of bacitracin resistance, which could point to

the existence of a cross-resistance phenotype with other antibiotics. Bacitracin resistance is spread among many bacteria and the genes encoding these mechanisms of resistance have also been identified. In the particular case of enterococci, rates of bacitracin resistance vary from country to country and between different species and environments [5]. A recent study involving enterococcal isolates from chickens and pigs collected in eight European countries revealed 89% resistant isolates [7]. A similar study with broiler strains from New Zealand reported 98.7% bacitracin-resistant enterococci [4]. In 2005, Lopes et al. [8] reported 90% and 65% of enterococcal isolates resistant to bacitracin from clinical and food environments, respectively. In enterococci, two mechanisms of resistance to bacitracin were described to be encoded in the same operon, the *bcrABD* operon [16]. In the only previously published study, only two wild-type genotypes were addressed [16]: the absence of genes, which fixed a phenotype of susceptibility; and the presence of all *bcr* genes, including the regulator *bcrR*, which fixed a phenotype of HLBR, corresponding to an MIC > 256 mg/L.

The MIC values for isolates in the current study are presented in Table 2. Nearly all clinical isolates showed HLBR, but this phenotype was detected in all environments, namely food, water and sand. Among the 58 isolates, 29 (50%) showed HLBR; 88% and 89% of veterinary and hospital isolates, respectively, presented HLBR. In contrast, environmental and food isolates presented a more broad range of MIC values for bacitracin, with only 38% and 29% of isolates, respectively, with HLBR. Among eight veterinary isolates, only one had an MIC < 64 mg/L, and all isolates from hospitals presented MIC values for bacitracin > 64 mg/L. Moreover, 35% of the food isolates tested presented MIC values for bacitracin <64 mg/L and 50% of environmental

behaved the same way. Similar resistance rates have been reported by others, showing that in Europe 40% of isolates had an MIC \geq 128 mg/L [7], which is comparable with our value of 53%. In New Zealand, these numbers are much higher, and Manson et al. [4] reported 99% of isolates with HLBR. This huge difference in HLBR percentages between European countries and New Zealand may be related to the fact that bacitracin was not banned from animal feeding in New Zealand.

Results of the screening of the *bcr* operon are also presented in Table 2. The *bcrA* and *bcrB* genes, coding for the ABC transporter that mediates bacitracin efflux, were the most disseminated, being detected simultaneously in 46 of the 58 isolates tested. These two genes were detected in all veterinary and hospital isolates. Likewise, *bcrA* and *bcrB* were detected in all isolates from dairy products, with the exception of one isolate for which the *bcrB* gene was not detected. Despite being disseminated in all environments, these two genes were only present simultaneously in 36% of sand isolates and in 80% of water isolates. Among the *bcr* structural genes, *bcrD* is the less disseminated, being found in only 7 of the 58 isolates tested, 6 of which were identified as *E. faecalis*. This gene was not detected in isolates from dairy products and sand. The transcriptional activator *bcrR* is also present in all environments, being detected in 32 (55%) of the isolates. Although less disseminated than *bcrAB* genes, *bcrR* often appears associated with these two genes. The complete *bcr* operon was detected in only five isolates from veterinary and hospital origin. All of them were HLBR and identified as *E. faecalis*. Among the veterinary isolates four genotypes were detected: *bcrAB*; *bcrABD*; *bcrABR*; and *bcrABDR*. Isolates with HLBR belonged to four different species, *E. faecalis*, *E. faecium*, *Enterococcus avium* and *Enterococcus gallinarum*. The complete operon was

detected in three of the hospital isolates and the other genotypes present were *bcrAB* and *bcrABR*. The only *E. faecium* hospital isolate had the genotype *bcrABR*. In dairy products *bcr* genes were found in five different species, *E. faecalis*, *E. faecium*, *E. hirae*, *Enterococcus durans* and *Enterococcus raffinosus*. In all except one of these isolates the presence of *bcrA* and *bcrB* simultaneously was detected, and the *bcrR* gene was absent in 53% of the dairy isolates. Water enterococcal isolates were all identified either as *E. faecalis* or *E. faecium*. The *bcrABR* genotype was detected in 60% of these isolates, all except one of which were HLBR. Isolates from sand showed greater diversity of genotypes, associated with only three species, *E. faecalis*, *E. faecium* and *E. hirae*. *bcr* genes were absent in two of the sand isolates tested and their MIC values for bacitracin were 8 mg/L and 24 mg/L for *E. hirae* and *E. faecalis*, respectively.

As mentioned above, the *bcrD* gene was only detected in seven isolates. Six of these isolates, identified as *E. faecalis*, presented the four *bcr* genes and were HLBR. The fact that they were all collected from clinical and veterinary infections may be relevant. Although no longer used in animal feeding, bacitracin is currently used in some topical preparations both in human and veterinary medicine. Therefore, our results somehow suggest that bacitracin use in medicine may exert a selective pressure for the spread of the *bcrABDR* genotype in the clinical and veterinary setting. This aspect is very important and, although not the aim of our work, requires further elucidation. Future work regarding the reasons for the increase in HLBR in *E. faecalis* should also address cross-resistance and selective pressure from other polypeptides used for the same purposes as bacitracin, as well as the presence of both HLBR and the *bcr* resistance genes from *E. faecalis* in strains from other genera, namely *Streptococcus* and

Staphylococcus collected from the same environments. These bacteria, namely *S. pneumoniae* and *S. aureus*, can carry the *bacA* gene, which is homologous to *E. faecalis bcrD*, and are also exposed to topical bacitracin used for treatment of skin and oropharyngeal infections. If HLBR is to be found in these species, the use of bacitracin in medicine may need to be re-evaluated, as it was in animal feeding. Work regarding these aspects is being established at the moment.

All 29 isolates showing HLBR had both *bcrA* and *bcrB* genes. Among these, three genotypes were detected, namely *bcrAB*, *bcrABR* and *bcrABDR*, in 21%, 62% and 17% of the isolates, respectively, suggesting that in the absence of *bcrD* and/or *bcrR* an HLBR phenotype is still possible. To understand the role of the *bcrR* gene in the HLBR phenotype, two strains with HLBR were selected, one bearing the *bcrAB* genotype (*E. faecalis* strain 1213) and the other carrying the *bcrABR* cluster of genes (*E. hirae* strain LN85). Both strains were grown in the absence and presence of bacitracin corresponding to 0.25× MIC (64 mg/L). The results are presented in Fig. 1. Strain 1213 shows similar growth in the presence and absence of bacitracin (Fig.1A). However, strain LN85, carrying the regulator gene *bcrR*, which responds to the presence of bacitracin [17], showed a delay in the growth curve in the presence of bacitracin, although it managed to reach the same final OD as in the absence of the antibiotic (Fig.1B). This behaviour suggests that *bcrR*, and the genes under its control, are induced in the presence of bacitracin, thus leading to a delay in growth. It also suggests that in the absence of the *bcrR* gene, the expression of *bcrAB* genes might be constitutive, thus explaining the absence of a delay in growth in the presence of

bacitracin. In fact, this hypothesis appears to be correct, since RT-PCR results (Fig. 2) demonstrate clearly that *bcrAB* genes are constitutively expressed in the absence and presence of the antibiotic. In strain LN85 carrying the *bcrR* gene, the expression of *bcrAB* is inducible by bacitracin, as expected from the work of Gauntlet et al. [17].

Gauntlet et al. [17] have recently determined the nucleotides in the promoter region of *bcrA* that are essential for BcrR binding and consequent regulation of the *bcrABD* operon. We also decided to investigate the intergenic sequence upstream from *bcrA* in both strains LN85 and 1213 and to compare it with the published sequence [17]. We also sequenced the same region in *E. faecalis* strain B23897 carrying the *bcrABDR* genotype and also expressing HLBR. Results are presented in Fig. 3 (top). The nucleotides essential for BcrR binding and regulation are conserved in both strains LN85 and B23897, allowing one to assume that the mechanism of BcrR regulation of the *bcrAB* (in LN85) and *bcrABD* (in B23897) operons is the same as that proposed by Gauntlet et al. [17]. This finding is in accordance with the induction of *bcrAB* genes observed when strain LN85 was grown in the presence of bacitracin. Figure 3 (bottom) shows the sequence of the region upstream from *bcrA* in strain 1213, which lacks *bcrR*. The nucleotides essential for binding of BcrR are absent from this sequence. No insertion sequence or deletion was detected in this sequence and a BLAST survey revealed no homologies to any sequences in the database. This suggests that the *bcrABDR* and *bcrABR* (this one could have derived from *bcrABDR* by a deletion event of the *bcrD* gene) clusters may have a different origin from that of the transporter alone, encoded by *bcrAB*. In fact, the cluster described by Manson et al. [16] and the other we

report here, *bcrABR*, were only detected in isolates from a clinical setting. In contrast, the transporter, encoded by *bcrAB* genes, was detected in environmental isolates. There might be a different origin for those operons: first, for the *bcr* operons under the regulation of the *bcrR* gene, which has been selected by pressure in the clinical setting; and second, for the ABC type of transporter, disseminated in the environmental non-clinical isolates. This hypothesis needs further studies to be confirmed.

Despite the abovementioned results pointing to the hypothesis that *bcrAB* genes are necessary and sufficient for expression of a HLBR phenotype, there are eight isolates, carrying either the *bcrAB* or the *bcrABD* genotypes, showing an MIC < 64 mg/L. Only one of these isolates comes from the clinical environment. Preliminary studies with these isolates (results not shown) point to the presence of mutations on both BcrA and BcrB proteins, which may account for significant changes in structure/function of the BcrAB transporter. This hypothesis needs further studies (ongoing work) but could explain the absence of the HLBR phenotype in the presence of *bcrAB* genes.

The *bcr* genes and the HLBR phenotype were detected in different species. However, with the exception of the *bcrD* gene, for which association with *E. faecalis* species is clear, dissemination of the other genes appears to be independent of the species. Our work does not reveal any correlation between bacitracin resistance and enterococcal species. However, previous work has suggested that *E. faecalis* is more susceptible to bacitracin compared with *E. faecium* [5].

In summary, our work provides for the first time a quantitative analysis of the spread of bacitracin resistance among isolates of the genus *Enterococcus*. MIC values were determined and correlated with the presence/absence of *bcrABDR* resistance genes. HLBR appears to be associated with clinical environments and future research should address the role of the use of both bacitracin and other polypeptides in selection of this resistance. However, HLBR was found also in strains from food, water and sand. This high-level resistance to bacitracin was detected in several species, namely *E. faecalis*, *E. faecium*, *E. durans*, *E. hirae*, *E. avium* and *E. gallinarum*, revealing that bacitracin resistance genes are not confined to the most common species associated with clinical environments. The BcrAB ABC transporter was shown to be the most disseminated mechanism of bacitracin resistance and is sufficient to confer HLBR. This phenotype is also found associated with other genotypes, namely *bcrABR* and *bcrABDR*. The *bcrD* gene, clearly associated with the species *E. faecalis*, appeared always together with the ABC transporter mechanism. Results from our laboratory (results not shown) suggest that it may constitute an additional resistance mechanism, which is relevant in case the ABC transporter is shut-off. Finally, our work suggests that there may be two different genotypes associated with bacitracin resistance in *Enterococcus*, which may have evolved separately, one in which the *bcr* genes are under the regulation of the BcrR protein and the other relying only on the transporter encoded by *bcrAB* genes.

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Fig. 1. Growth curves for (A) *Enterococcus faecalis* strain 1213 carrying the *bcrAB* genotype and (B) *Enterococcus hirae* strain LN85 carrying the *bcrABR* genotype in the absence of bacitracin (◆) and in the presence of 64 µg/ml of bacitracin (▲). OD, optical density.

Fig. 2. A 1% agarose gel of reverse transcriptase polymerase chain reaction (RT-PCR) products obtained from *Enterococcus faecalis* strain 1213 and *Enterococcus hirae* strain LN85. +, RNA extracted from cells grown in the presence of bacitracin; –, RNA extracted from cells grown in the absence of bacitracin; control, PCR products obtained from RNA to which no reverse transcriptase enzyme was added during the reaction.

Fig. 3. (Top) Scheme of the *bcrABDR* cluster, as published by Manson et al. [16]. The sequence of the *bcrR–bcrA* intergenic regions in *Enterococcus hirae* LN85 and *Enterococcus faecalis* B23897 is presented. The start of BcrA and the end of BcrR proteins are marked, as well as the –35, –10 and RBS regions in the *bcrA* promoter. Grey dotted line marks the nucleotide sequence essential for the maximum expression of the genes [17]. Grey line marks the inverted repeat sequences that are essential for BcrR binding. Inside these sequences, a double grey line marks the nucleotides to which BcrR binds, as demonstrated by Gauntlet et al. [17]. (Bottom) Scheme of the organisation of the *bcrAB* genes as determined for strain 1213. The sequence of the amplicon obtained from polymerase chain reaction with primers 9 and 10 is presented. No similarities were found this between sequence and the one presented above (top figure).

Table 1

Primers used to amplify each of the *bcrABDR* genes and the region upstream from *bcrA* in strains lacking *bcrR* (see Fig. 3)

Gene	Primer	Sequence	Amplicon size (bp)
<i>bcrA</i>	1	5'-CCGCAATGAAAATGATGTTG-3'	584
	2	5'-TGCGGCTATCTTACCATCTG-3'	
<i>bcrB</i>	3 ^a	5'-AAAGAAACCGACTGCTGATA-3'	489
	4	5'-GCTTACTTGTATAGCAGAGA-3'	
<i>bcrD</i>	5	5'-GCGAAGCGTTTAAGGAAATG-3'	482
	6	5'-TGGCACAGCAAGAAAGAATG-3'	
<i>bcrR</i>	7	5'-TAACGCAGGAACAACCTTGC-3'	461
	8	5'-CAAAGCGGTAATGGTGAGG-3'	
IR ^b	9	5'-AGAATTCCTGCACTTCACG-3'	558
	10	5'-CAGATGGTAAGATAGCCGCA-3'	

^a Sequence published by Manson et al. [16].

^b Intergenic region between *bcrA* and the transposase.

Table 2

Minimum inhibitory concentration (MIC) and *bcr* genotype for each of the studied *Enterococcus* isolates, according to their origin and species

Origin	Isolate	Species	MIC (mg/L)	Genotype				
				<i>bcrAB</i>	<i>bcrABD</i>	<i>bcrABR</i>	<i>bcrABDR</i>	Other ^a
Veterinary	337/03	<i>E. faecium</i>	24		x			
	1213/02	<i>E. faecalis</i>	>256	x				
	1903/02	<i>E. gallinarum</i>	>256	x				
	2151/03	<i>E. faecalis</i>	>256				x	
	2479/02	<i>E. faecalis</i>	>256			x		
	3031/03	<i>E. faecalis</i>	>256				x	
	3179/03	<i>E. faecium</i>	>256			x		
	3671/03	<i>E. avium</i>	>256	x				
	Hospital	F13206	<i>E. faecalis</i>	128	x			
19015		<i>E. faecium</i>	>256			x		
111724		<i>E. faecalis</i>	>256			x		
118103		<i>E. faecalis</i>	>256				x	
A33112		<i>E. faecalis</i>	>256			x		
A33562		<i>E. faecalis</i>	>256				x	
B23897		<i>E. faecalis</i>	>256				x	
B24452		<i>E. faecalis</i>	>256	x				
RG92		<i>E. faecalis</i>	>256			x		
Diary products	QCB7	<i>E. hirae</i>	8	x				
	QN20	<i>E. hirae</i>	16	x				
	QCB14	<i>E. faecalis</i>	16	x				
	LA13	<i>E. durans</i>	24	x				
	QSE118	<i>E. faecalis</i>	24	x				

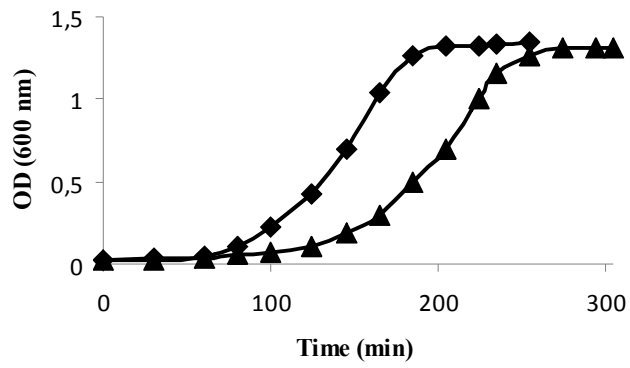
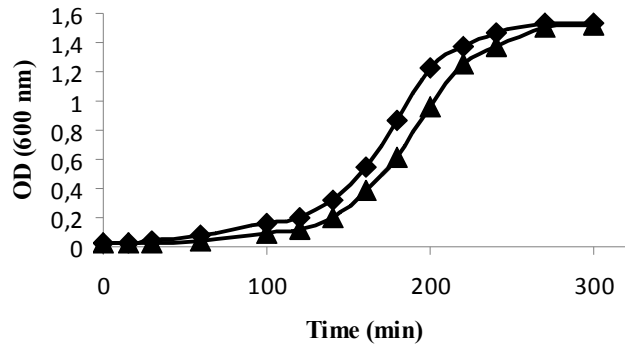
	QA40	<i>E. raffinosus</i>	48			x	
	LA77	<i>E. hirae</i>	64				x
	QA27B	<i>E. faecium</i>	64	x			
	LA41	<i>E. faecium</i>	96			x	
	LCB18	<i>E. faecalis</i>	96			x	
	QA34	<i>E. hirae</i>	96	x			
	LA34	<i>E. durans</i>	128			x	
	LA19	<i>E. faecalis</i>	>256	x			
	LA160	<i>E. durans</i>	>256			x	
	LCB3	<i>E. faecium</i>	>256			x	
	LN85	<i>E. hirae</i>	>256			x	
	QN10	<i>E. durans</i>	>256			x	
Water	SMAS 3	<i>E. faecalis</i>	3				x
	SMAS 9	<i>E. faecalis</i>	4				x
	755/03	<i>E. faecalis</i>	6		x		
	1007/02	<i>E. faecalis</i>	12	x			
	754/06	<i>E. faecium</i>	48			x	
	754/01	<i>E. faecium</i>	>256			x	
	754/04	<i>E. faecalis</i>	>256			x	
	782/02	<i>E. faecium</i>	>256			x	
	SMAS 1	<i>E. faecalis</i>	>256			x	
	SMAS 10	<i>E. faecalis</i>	>256			x	
Sand	5LM-5	<i>E. hirae</i>	0.75				x
	22-D2	<i>E. faecalis</i>	3				x
	21-D2	<i>E. hirae</i>	4				x
	S1-11	<i>E. faecalis</i>	4				x
	22-D5 ^b	<i>E. hirae</i>	8				x
	14D-1 ^b	<i>E. faecalis</i>	24				x

S1-6	<i>E. faecalis</i>	48		x
14D-2	<i>E. faecium</i>	64		x
9LM-1	<i>E. faecium</i>	64		x
9LM-10	<i>E. faecalis</i>	64		x
1LM-2	<i>E. hirae</i>	>256	x	
5LT-7	<i>E. faecium</i>	>256		x
8LT-2	<i>E. faecium</i>	>256		x
8LT-4	<i>E. faecium</i>	>256		x

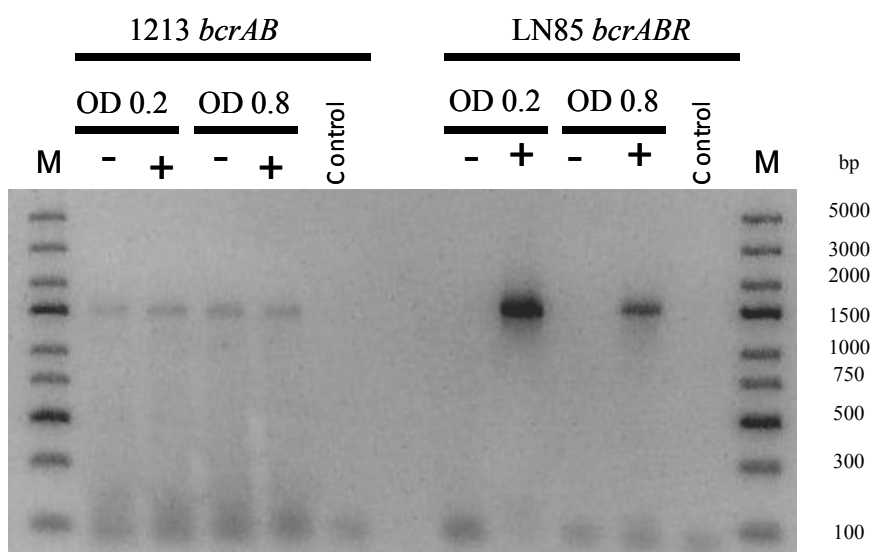
^a Other genotypes were detected in some strains, namely *bcrA* only, *bcrB* only, *bcrAR* and *bcrBR*, which do not code for a complete BcrAB transporter and are thus irrelevant for bacitracin resistance analysis.

^b These strains do not carry any *bcr* genes.

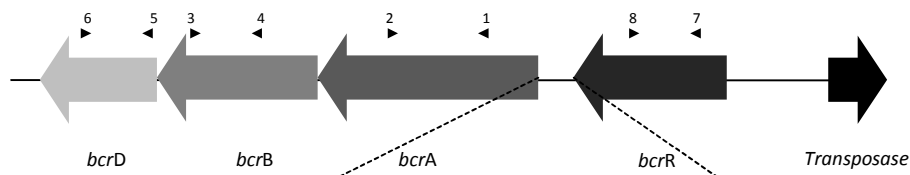
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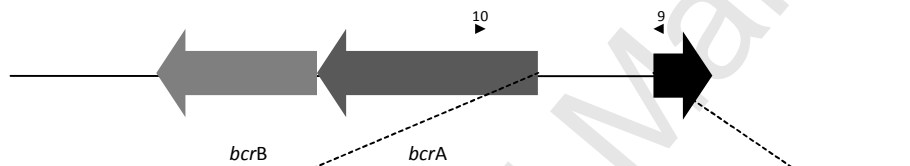
B



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AATCCTTTCTGTAATGCTGTTTGTGCAGACCACAATTTCAATTATCTGTCACCACA
bcrA RBS -10
 TAGCAAAAGCCAAAGAAACCTACCGTCACAATGCTGACACAATAGATGTCAGCATG
 -35
 AAAAAAGCTCCGACACTTATCGTGTCAGAGCCTATTTTCAAGGTTTTATGAT
bcrR



bcrA
 GTTTTGTAAGATTTTCTGTTTCAATCACATATCCATAATCATCAACAGAACAAAGAGCTCCACACCCTTTTGCAGCCAGATTTGCTTACGGCT
 TGGGACGATTTGACGCAGATGATTGAATTGCGTATTCATGAGCAAAGTTTGGCACACCGCAAACAAGGGTAAGAGCTATGAACTGAAATAC
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