

## NOTE / NOTE

## Identification of novel *horA*-harbouring bacteria capable of spoiling beer

Monique Haakensen and Barry Ziola

**Abstract:** An ATP-binding cassette (ABC) multi-drug resistance (MDR) gene was found in 4 Gram-positive bacterial isolates of environmental origin and found capable of spoiling beer. The bacteria isolated were *Bacillus cereus*, *Bacillus licheniformis*, *Paenibacillus humicus*, and *Staphylococcus epidermidis*; all of which were previously unappreciated as beer-spoilage bacteria. The MDR gene found in these bacteria has less than 37% similarity to known ABC MDR proteins described for *Bacillus* and *Staphylococcus*, and this is the first finding of an ABC MDR gene in the genus *Paenibacillus*. The sequenced region of the gene was translated and compared phylogenetically with the closest GenBank matches of the respective species and the closest GenBank matches overall. The ABC MDR proteins from these isolates were found to cluster among known sequences of HorA, sharing 99.5% identity within the sequenced region. In the beer-spoilage-associated genera *Lactobacillus* and *Pediococcus*, the presence of the MDR gene *horA* correlates with the ability to grow in beer. As the unique *horA*-harbouring isolates described here are capable of growing in beer, it is likely that the presence of the *horA* gene likewise confers hop resistance to these organisms.

**Key words:** beer-spoilage bacteria, *Firmicutes*, *horA*.

**Résumé :** Un gène de résistance multiple aux drogues (MDR) à cassette ABC (ATP binding cassette) a été trouvé chez quatre isolats bactériens Gram-positifs d'origine environnementale, capables d'altérer la bière. Les bactéries isolées étaient *Bacillus cereus*, *Bacillus licheniformis*, *Paenibacillus humicus* et *Staphylococcus epidermidis*, lesquelles avaient été sous-estimées en regard de leur potentiel d'altération de la bière. Le gène MDR de ces bactéries possède moins de 37 % de similarité avec les protéines MDR ABC connues chez *Bacillus* et *Staphylococcus*. Il est aussi le premier gène MDR ABC identifié chez le genre *Paenibacillus*. La région séquencée du gène a été traduite et comparée d'un point de vue phylogénique aux séquences les plus apparentées de ces espèces respectives dans GenBank, ainsi qu'aux séquences les plus apparentées en général dans GenBank. Les protéines MDR ABC de ces isolats s'agrègent avec les séquences connues de HorA, partageant 99,5 % d'identité à l'intérieur de la région séquencée. Chez les genres *Lactobacillus* et *Pediococcus*, qui sont associés à l'altération de la bière, la présence du gène MDR *horA* est corrélée avec leur capacité de croître dans la bière. Puisque les isolats uniques comportant *horA* décrits ici sont capables de pousser dans la bière, il est probable que la présence du gène *horA* leur confère aussi la résistance au houblon.

**Mots-clés :** bactéries d'altération de la bière, *Firmicutes*, *horA*.

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Efflux pumps of the ATP-binding cassette (ABC) multi-drug resistance (MDR) type are commonly used by Gram-positive organisms to counter the activity of antimicrobial compounds. Here we report the finding of a new ABC MDR gene for the genera *Bacillus*, *Paenibacillus*, and *Staphylococcus*. Phylogenetic analysis of the novel gene indicated that it is homologous to the hop resistance gene *horA* (Sami et al. 1997). The finding of *horA* is of consequence to the brewing industry, as it has been shown that the presence of *horA* is highly correlated to the ability of *Lactobacillus* and *Pediococcus* isolates to grow in beer

(Haakensen et al. 2007, 2008). Like other *horA*-positive isolates identified to date, these 3 genera are Gram-positive and belong to the highly diverse phylum *Firmicutes*. Bacteria of this phylum are known to cause more than 90% of beer-spoilage incidents (Sakamoto and Konings 2003). While *Bacillus* and *Staphylococcus* isolates are sometimes found in beer, they were not previously believed to be capable of growth owing to the presence of hop compounds (Campbell 2001; Priest and Campbell 2002), and *Paenibacillus* isolates have never been associated with beer spoilage. *Bacillus* and *Paenibacillus* species are responsible for numerous food-

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poisoning incidents, are capable of withstanding temperature fluctuations, and are readily introduced to grain-based foods owing to their natural occurrence in soil (Pirttijärvi et al. 2000; Granum 2001), while *Staphylococcus* species are implicated in a variety of human and animal diseases. Thus, each of the *horA*-positive bacteria identified here is ubiquitous and can be easily transported into the brewery environment. Since these bacteria were isolated as environmental contaminants of 2 types of spoiled home-brewed beer, their carriage of *horA* is a novel finding not only of relevance to the brewing industry but also to other fermentation industries and possibly to human health and animal husbandry.

Two varieties of spoiled home-brewed beer (one light and one dark, originating from 2 different kit manufacturers) were cultured on de Man, Rogosa, Sharpe (MRS) agar plates containing 10 ppm actidione to inhibit growth of yeast. Incubation was at 30 °C in a candle jar. Four morphologically distinct colonies were picked and inoculated into MRS broth at 30 °C. Bacterial DNA was extracted as described previously (Haakensen et al. 2007). The first 3 variable regions of the 16S rRNA gene were amplified by polymerase chain reaction (PCR) and sequenced as described by Dobson et al. (2002). The NCBI GenBank Basic Local Alignment Search Tool (Altschul et al. 1990) was used to determine the identity of each isolate, and in all cases the best GenBank matches had an E-value of zero. The identities found as the best match for each novel isolate were *Bacillus cereus*, *Bacillus licheniformis*, *Paenibacillus humicus*, and *Staphylococcus epidermidis* (isolate Nos. MH1–4, respectively). The sequenced region of the 16S rRNA gene amplified from each isolate was deposited in GenBank under the accession Nos. EU091076, EU091078, EU091079, and EU091077, respectively.

Each of the 4 isolates was capable of growing in commercially available beer. Two beers were used in growth experiments. Beer 1 was a filter-sterilized 4% (v/v) alcohol beer, pH 5.2, containing an average of 9.8 bitterness units. Beer 2 was a pasteurized 5% (v/v) alcohol beer, pH 4.8, containing an average of 11.0 bitterness units. Cultures were grown in 15 mL capped tubes containing modified de Man, Rogosa, Sharpe (MMRS) broth containing incremental amounts of beer before being subcultured into Beer 1 and Beer 2. The incremental concentrations of beer used were 50% Beer 2 : 50% 2× MMRS, followed by 85% Beer 2 : 15% 2× MMRS (85:15 medium). Bacteria were grown at 30 °C.

In the brewing industry, the onset of turbidity is synonymous with bacterial growth; therefore, turbidity is used as an indicator of product spoilage by bacteria. As such, once turbidity could be seen in beer, 500 µL from each tube of beer was inoculated into fresh tubes of 85:15 medium to confirm that turbidity was the result of bacterial growth. This step was also performed on control tubes of beer, which did not produce visible turbidity after 21 days, to ensure that there was no bacterial contamination present in the commercially available beer being used in the growth assay. The novel isolates grew in 85:15 medium when subcultured from the initial beer cultures. The bacteria were then subcultured from the first beer cultures into a second set of tubes containing beer. This was done to ensure that growth seen in the initial beer cultures was not due to a carryover of MMRS nutrients from the preceding growth of bacteria in

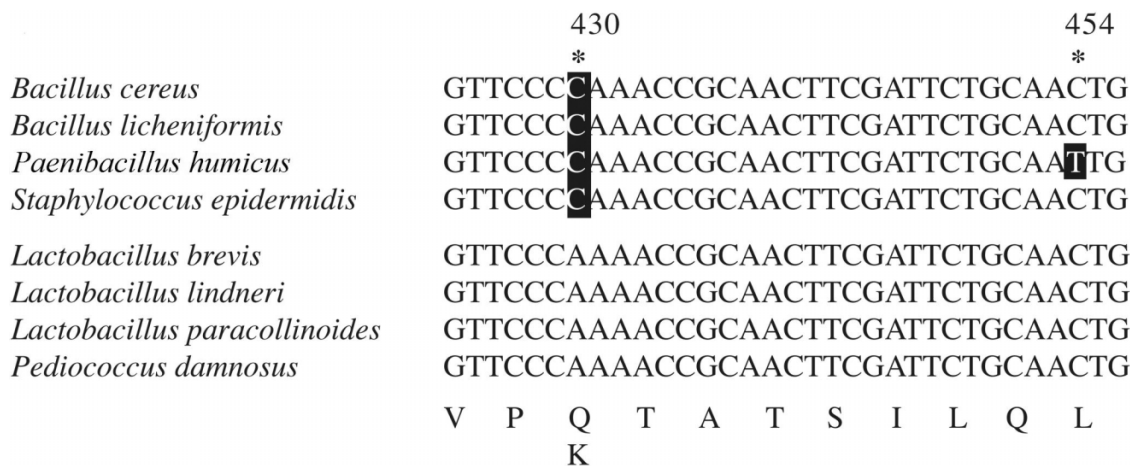
85:15 medium. This second set of beer cultures was then also assessed for bacterial growth in the same manner as the first set. Bacterial isolates producing visible turbidity in the second culture in beer (and subsequent growth upon sub-culture to 85:15 medium) were considered to be hop resistant and capable of growing in beer. All 4 novel isolates tested were capable of growing in both Beer 1 and Beer 2. Of the 4 isolates, *B. licheniformis* grew the fastest in beer, producing visible turbidity in only 14 days. Both *B. cereus* and *S. epidermidis* grew in 18 days, while *P. humicus* took 20 days to produce visible turbidity in beer.

As the novel isolates were capable of growing in both home-made and commercially available beer, the isolates were tested for the presence of the *horA* gene, which has previously been shown to correlate with the ability of related bacterial genera to grow in beer (Haakensen et al. 2007, 2008). The *horA* gene was tested for by PCR pre- and post-growth in beer as described previously (Haakensen et al. 2007, 2008). For each isolate, *horA* PCR amplicons were produced and then sequenced to determine similarity within the amplified region (Fig. 1). *horA* PCR amplicons for pre- and post-growth samples of each isolate were found to be identical. The sequenced region corresponded to the PCR-targeted bases 318–521 (coding for amino acids 106–173) of the *horA* gene, including most of the region between the second and third transmembrane helices that is believed to be important to substrate binding specificity (Priest and Campbell 2002). The NCBI GenBank database was screened for genes and proteins with homology to the ABC MDR gene amplified from these bacteria; however, the only significant matches were to *horA* or HorA from known beer-spoilage bacteria in the genera *Lactobacillus* and *Pediococcus*. The sequences of the *horA* homologous amplicons were deposited in GenBank under the accession Nos. EU091080 (*B. cereus*), EU091082 (*B. licheniformis*), EU091083 (*P. humicus*), and EU091081 (*S. epidermidis*).

It should be noted that 16S rRNA gene and *horA* PCR amplicons were sequenced from each organism originally isolated from spoiled beer and again after the same organism had been grown in commercial beer to ensure that the amplified *horA* homologous gene had indeed originated from each of the novel bacteria and not arisen because of PCR contamination by DNA from another *horA*-positive organism. The *horA* gene was PCR amplified in multiplex with the 16S rRNA gene as previously described (Haakensen et al. 2007), and both amplicons were visualized by agarose gel electrophoresis, isolated, and sequenced from both directions. As the *horA* and 16S rRNA genes were amplified in multiplex, the amplified *horA* gene could only originate from the organism identified by the corresponding 16S rRNA gene sequence. For each of the 4 novel isolates, the *horA* and 16S rRNA amplicons for the organism isolated from spoiled beer and for organism after growth in commercial beer had sequences identical to the sequences now deposited in GenBank. As *horA* was previously shown to be highly correlated with the ability of related genera to grow in beer (Haakensen et al. 2007, 2008), the clear association of *horA* with each of these 4 novel beer-spoilage isolates strongly suggests that the presence of *horA* is also associated with hop resistance in these bacteria.

Sequences corresponding to the *horA* gene of known beer-

**Fig. 1.** Multiple sequence alignment of bases 424–456 of *horA* from the 4 novel *horA*-harbouring bacteria and GenBank *horA* sequences (numbering starting from the start codon of *Lactobacillus brevis horA* gene AB005752). *Lactobacillus brevis*, AB005752; *Lactobacillus lindneri*, AB167898; *Lactobacillus paracollinoides*, AB178589; *Pediococcus damnosus*, AB218963.



spoilage bacteria and that were accessible through GenBank were compared with the novel ABC MDR sequences obtained from the 4 unique isolates. The GenBank search revealed that there are no currently known *Bacillus* or *Staphylococcus* genes yielding proteins with >37% identity with *horA* and that no ABC MDR gene has been previously reported for any *Paenibacillus* species. Interestingly, the *Paenibacillus* isolate described here was identified as *P. humicus*, a newly described species, about which little is yet known, and it is possible that other *P. humicus* isolates may also possess ABC MDR genes. The ABC MDR sequences amplified from *B. cereus*, *B. licheniformis*, *P. humicus*, and *S. epidermidis* in this study were compared with GenBank sequences for *horA* from known beer-spoilage bacteria and with the closest GenBank matches for ABC MDR genes from the respective species. These sequences were compiled and used to create a multiple sequence alignment using ClustalX (Thompson et al. 1997). The alignment was truncated using the GeneDoc program (Nicholas et al. 1997).

The 203 bp region from the ABC MDR gene of *B. cereus*, *B. licheniformis*, *P. humicus*, and *S. epidermidis* that we analyzed had <37% identity with known ABC MDR genes of these genera, yet shared >99% identity with *horA* from beer-spoilage bacteria. The multiple sequence alignment in Fig. 1 shows the region of the *horA* amplicon from the novel isolates containing base changes in comparison with known *horA* genes. The sequenced region of each of the 4 novel isolates possessed a single base change at position 430, resulting in an amino acid change from lysine to glutamine. The *P. humicus* isolate also possessed a null mutation at base 454. This exceptionally high level of conservation is indicative of lateral transfer of genetic material and the existence of environmental selective pressure(s) for *horA* among Gram-positive bacteria.

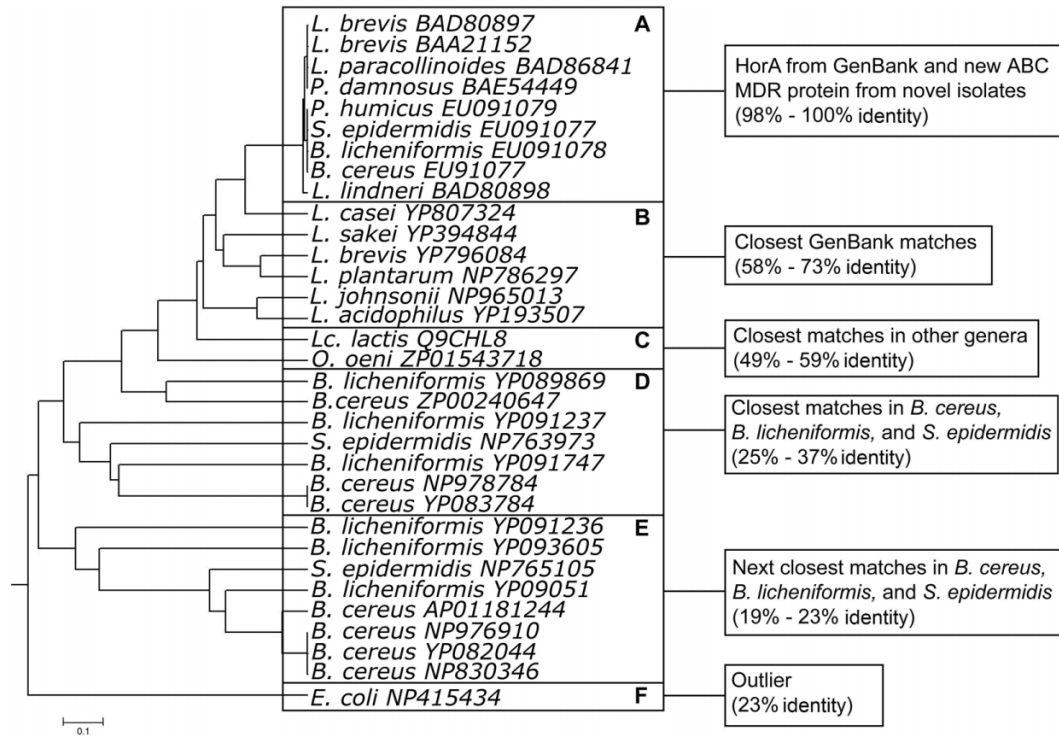
Phylogenetic analysis was performed to confirm the identity of the ABC MDR gene amplified from the 4 novel isolates in this study. Figure 2 is a phylogenetic tree of the protein sequence corresponding to the PCR-amplified region of the ABC MDR gene. The phylogenetic tree was created using the Unweighted Pair Group Method with Arithmetic

mean algorithm in the MEGA2 software program (Kumar et al. 2001). While the tree in Fig. 2 was created from a truncated portion of the ABC MDR proteins, the branching of this tree is identical to the phylogenetic trees created when full-protein or DNA sequences of the same GenBank sequences are used with bootstrap replicates set at 1000 (data not shown). That is, when only GenBank sequences are used (i.e., the full-length protein or DNA sequences of the GenBank sequences included in Fig. 2, but not our newly identified sequences), the resulting tree has the same branching pattern. This goodness-of-fit test indicates that the 203 bp region sequenced from the ABC MDR gene of our newly identified beer-spoilage isolates is appropriate for determining the identity of the gene. Tree structure and groupings are also identical when various phylogenetic analysis methods are used; i.e., Neighbor-Joining, Minimum Evolution, or Maximum Parsimony (data not shown). The phylogenetic clades outlined in Fig. 2 show that the sequenced region of the ABC MDR gene found in our *B. cereus*, *B. licheniformis*, *P. humicus*, and *S. epidermidis* isolates is >99% similar to *HorA* and <37% similar to any known ABC MDR proteins in the respective species. Information from the goodness-of-fit test and the high percentage of identity between the sequenced region of the ABD MDR gene from our novel beer-spoilage isolates and *horA* together indicate it is extremely likely that the ABC MDR gene found in the 4 novel isolates is, in fact, *horA*.

The new finding of *horA* in isolates belonging to the genera *Bacillus*, *Paenibacillus*, and *Staphylococcus* suggests that *horA* exists in bacteria in environments beyond the brewery. The finding of *horA* in lactobacilli originating from human vaginal flora or feces or from various fermentations (corn silage, fuel ethanol, kefir, moto, and wine) provides support for this suggestion (Haakensen et al. 2007), while the free exchange of genetic material among *Firmicutes* provides an impetus for investigating whether *horA* is present in a wider range of bacterial genera and environments.

In summary, our findings have 2 major implications. First, novel bacteria with the potential to spoil beer exist and, since these bacteria are not currently being tested for as

**Fig. 2.** Phylogenetic tree of amino acids 106–173 from HorA, the corresponding region of the new ATP-binding cassette (ABC) multi-drug resistance (MDR) protein from the 4 novel isolates, and the corresponding region of the closest GenBank protein matches. (A) HorA sequences from GenBank and new ABC MDR protein from the 4 novel isolates (98%–100% identity). (B) Closest GenBank matches; all are putative ABC MDR proteins identified through genome sequencing projects (58%–73% identity to HorA). (C) Closest matches to proteins from other genera (49%–59% identity to HorA): *L. lactis* (*Lactococcus lactis* LmrA protein), *O. oeni* (*Oenococcus oeni* OmrA protein). (D) Closest matches within *Bacillus cereus*, *Bacillus licheniformis*, and *Staphylococcus epidermidis* (25%–37% identity to HorA). (E) Next closest matches within *B. cereus*, *B. licheniformis*, and *S. epidermidis* (19%–23% identity to HorA). (F) Outlier (23% identity to HorA), *E. coli* (*Escherichia coli* MsbA protein). Bar = 0.1 difference in 100 amino acids.



beer-spoilage organisms, present a threat to the brewing industry. Second, the *horA* MDR gene that is thought to be significant only in the context of brewing spoilage bacteria is also found in a range of ubiquitous bacteria that may play a role not only in fermentation industries but also in animal and human health.

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