Identification of *Brachyspira hyodysenteriae*-specific DNA fragments using representational difference analysis

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Abstract

Two novel *Brachyspira hyodysenteriae*-specific DNA fragments, designated as Bh100 and Bh400, were identified using representational difference analysis. To isolate the fragments the combined DNA of the *Brachyspira pilosicoli*, *Brachyspira intermedia*, *Brachyspira murdochii* and *Brachyspira innocens* reference strains was subtracted from the genome of *B. hyodysenteriae* strain B204. Both fragments were present in a single copy and mapped to different positions on the genome of *B. hyodysenteriae* B78T. Larger fragments encompassing the continuous open reading frames (ORF) of Bh100 and Bh400 were cloned and analysed. Whereas the ORF of 2130 bp encompassing Bh100 did not show homology to any known bacterial protein, Bh400 was part of a putative operon with significant homology to the phosphotransferase system of *Bacillus subtilis*. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Representational difference analysis; *Brachyspira hyodysenteriae*

1. Introduction

*Brachyspira hyodysenteriae* is the aetiological agent of swine dysentery (SD), a mucohaemorrhagic diarrhoea occurring worldwide and causing significant economic losses mainly due to retarded growth of convalescent pigs [1,2]. Treatment is hampered by a limited number of effective antibiotics and an increasing number of resistant strains [3], and vaccination with whole-cell bacterins is not effective [4]. Research concerning potential virulence factors of *B. hyodysenteriae* has focussed on motility [5] and motility-associated genes [6] as well as on different hemolysins (*tlyA* [7], *tlyB* and *tlyC* [8], and *hlyA* [9]). In addition, the NADH oxidase [10] and several surface-associated proteins have been identified [11], and a physical and genetic map of *B. hyodysenteriae* B78T has been constructed [12].

In addition to *B. hyodysenteriae*, four other *Brachyspira* species colonise the porcine intestine, namely *B. pilosicoli* [13], *B. intermedia*, *B. murdochii*, and *B. innocens* [14,15]. Although *B. pilosicoli* and *B. intermedia* can also cause disease, the clinical and histological picture is clearly different from that seen in SD [16]. As the molecular basis for the distinct pathogenicity of *B. hyodysenteriae* is not known we decided to isolate and characterise *B. hyodysenteriae*-specific genes. We hypothesised that these genes might encode previously unidentified virulence-associated factors which should, however, not be essential and, therefore, might present ideal targets for the future construction of live-attenuated vaccines.

2. Materials and methods

2.1. Bacterial strains, plasmids, primers and growth conditions

The bacterial strains, plasmids and primers used in this work are listed in Table 1. *Brachyspira* species were grown on Columbia agar (Oxoid, Wesel, Germany) in anaerobic jars using AnaeroGen sachets® (Oxoid, Basingstoke, England) at 42°C for 5–7 days. *Escherichia coli* strains were grown in Luria–Bertani medium supplemented with ampicillin (100 μg ml⁻¹) when appropriate.
Table 1

<table>
<thead>
<tr>
<th>Strains, plasmids and primers</th>
<th>Characteristics and source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. hyodysenteriae</em> B204</td>
<td>laboratory reference strain</td>
</tr>
<tr>
<td><em>B. pilosicoli</em> P436/78T</td>
<td>laboratory reference strain</td>
</tr>
<tr>
<td><em>B. innocens</em> P4</td>
<td>laboratory reference strain</td>
</tr>
<tr>
<td>B. intermedia</td>
<td>laboratory reference strain</td>
</tr>
<tr>
<td>C301</td>
<td>laboratory reference strain</td>
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<tr>
<td>12 field isolates</td>
<td>DNA sequence as well as the putative open reading frames (ORFs) were investigated for homologies in the GenBank/EMBL database.</td>
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</table>

2.2. Manipulation and analysis of DNA

Gel electrophoresis, Southern blot, transformations and PCR were done following standard protocols [17]. For pulsed-field gel electrophoresis (PFGE) bacteria were washed off the plates using PET IV solution (1 M NaCl, 10 mM Tris–HCl [pH 8], 10 mM Na₂EDTA), and DNA-imbedded agarose plugs were prepared as described previously [18]. Agarose plugs were cleaved with theendonuclease *Eag*I (isoischizomer to *Eco*I) and *Sal*I (both New England Biolabs, Bad Schwalbach, Germany), and were separated in a 1% agarose gel in 0.5

\[ 0.01 \text{MEDTA} \] using a CHEF-DR® III pulsed-field electrophoresis system (Bio-Rad, England Biolabs, Bad Schwalbach, Germany), and were separated using the settings: 6 V/cm, 12°C, block I from 7 to 12 s for 11 h and block II from 20 to 65 s for 13 h. For an improved separation of fragments with sizes between 300 and 1000 kb ramped switch times from 20 to 80 s for 24 h were used. Bh100 and Bh400 probes were prepared by PCR using *R.Bam*24 primer (Table 1) on the pCR®II-TOPO constructs; a *nox* gene-derived probe was prepared by PCR from *B. hyodysenteriae* B204 using primers Bnox1 and Bnox2 (Table 1). Probes were labelled with α-[³²P]dCTP using the random-priming method [19]. Hybridisations were performed overnight at 60°C in 6 x SSC, 0.5% (w/v) sodium dodecyl sulfate (SDS), 5 x Denhardt’s solution and 0.01 M EDTA [17]. Washing was done under low-stringency conditions (3 x SSC, 0.5% SDS, 65°C). Nucleotide sequencing was done by ‘primer walking’. Primers were purchased from MWG-Biotech AG (Ebersberg, Germany) and Invitrogen (Karlsruhe, Germany); the sequencing reactions were done by SeqLab (Göttingen, Germany). Sequencing data analyses was performed using the HUSAR 5.0 programme (dkfz, Heidelberg, Germany). The DNA sequence as well as the putative open reading frames (ORFs) were investigated for homologies in the GenBank/EMBL database.

2.3. Representational difference analysis (RDA) and isolation of RDA fragments

Chromosomal DNA of *Brachyspira* strains was pre-
pared as follows: Brachyspira were washed off the plates with TE-buffer (10 mM Tris [pH 8.0], 1 mM EDTA), centrifuged, resuspended in TE-buffer supplemented with 10 mM EDTA, 1% SDS, 500 μg ml⁻¹ Proteinase K, and incubated at 55°C for 1 h. Ribonuclease A (150 μg ml⁻¹) was added and incubation was continued at 37°C for 15 min. Proteins were extracted by adding of 1/5 volume of phenol equilibrated with TE-buffer (pH 7.8; Roti, Roth, Karlsruhe, Germany), freezing at −70°C for 1 h, adding 1/5 volume of chloroform:isoamyl (24:1) and centrifuging. The aqueous phase was removed, and the last two steps were repeated. The DNA was precipitated with isopropanol, washed with 70% ethanol, dried and resuspended in Aq. bidest.

The DNA was digested with the restriction enzyme DpnII (New England Biolabs, Bad Schwalbach, Germany), and an RDA was performed [20] using a modified protocol previously described [21]. Chromosomal DNA of B. hyodysenteriae B204 (2.5 μg) was used as ‘tester’ and a 10-, 100-, 1000-fold excess of the combined DNA of B. pilosicoli P43/6/78, B. intermedia AN26:93, B. mordochii C301, and B. innocens C336 was used as ‘driver’. After a single round of representation PCR products were cloned using the TOPO TA cloning kit® (Invitrogen, Groningen, The Netherlands). E. coli transformants containing B. hyodysenteriae-specific DNA were identified by differential Southern blotting of PCR products using the whole genomes of ‘tester’ and ‘driver’ as probes [21].

2.4. Cloning of DNA spanning the RDA fragments

Using the RDA fragments as probes, Southern blots were performed on B. hyodysenteriae DNA cut with a variety of restriction endonucleases in order to identify enzymes resulting in hybridising fragments of 2–6 kb in size. Two large DNA fragments (Bh106 and Bh400) were obtained by isolating and cloning a HindIII fragment of the appropriate size into pBluescript. One fragment (Bh104) was obtained using a reverse PCR approach; briefly, HindIII fragments of 4.5 kb were ligated into pBluescript. A PCR using primers M13rev and Bh100for (Table 1) was performed, the DNA was cut with PsI and HindIII and ligated into pBluescript. Subsequently colony blots using the RDA fragments as probes were performed to identify the correct E. coli transformants.

3. Results and discussion

3.1. Isolation of two B. hyodysenteriae-specific fragments using RDA

The RDA approach using B. hyodysenteriae B204 as tester and the reference strains of B. pilosicoli, B. innocens, B. intermedia and B. mordochii as driver, revealed two specific fragments of 487 and 175 bp in length. The fragments, designated as Bh100 and Bh400 respectively, reacted solely with the DNA of B. hyodysenteriae (Fig. 1). Fragments Bh100 and Bh400 were used as probes in Southern blots with 74 B. hyodysenteriae, 12 B. pilosicoli, six B. intermedia, five B. mordochii, and five B. innocens field isolates and all reference strains (Table 1). Both probes hybridised to a single band of different sizes in all 74 B. hyodysenteriae field isolates (Fig. 2A). Fragment Bh100 did not hybridise to any strain of the other species (Fig. 2B). Fragment Bh400 hybridised to a single B. pilosicoli field isolate (Fig. 2B); using high-stringency washing conditions (0.1×SSC, 0.5% SDS, 65°C) no positive reaction with strains of other species was observed (data not shown). As B. hyodysenteriae and B. pilosicoli are not closely related species [13] this hybridisation of a single B. pilosicoli field isolate to Bh400 is a first indication for the occurrence of horizontal gene transfer between B. pilosicoli and B. hyodysenteriae and might be caused by the generally transducing bacteriophage VSH-1 [22] or a related virus. In summary, these results show that we have isolated two novel B. hyodysenteriae-specific DNA-fragments using the RDA approach. We have shown that a mixture of four species can be used as ‘driver’ and, in addition, can

![Fig. 1. Identification of B. hyodysenteriae-specific DNA fragment Bh400 by Southern blot analyses. PCR products of cloned RDA fragments were prepared and hybridised with radioactive-labelled chromosomal DNA of the ‘driver’ (A) and the ‘tester’ (B); lane 1, B. hyodysenteriae-specific RDA fragment designated as Bh400; lanes 2–7, other non-specific RDA fragments; lane 8, positive control. The arrows indicate the position of Bh400 not hybridised by the driver (open arrow) and hybridised by the tester (solid arrow).](https://academic.oup.com/femsle/article-abstract/210/2/173/487872/download)
serve as DNA probe in a Southern blot, thereby allowing rapid identification of unique DNA sequences.

3.2. Localisation of Bh100 and Bh400 on the B. hyodysenteriae genome

In order to investigate whether the fragments Bh100 and Bh400 belong to a single B. hyodysenteriae-specific chromosomal locus they were mapped on the B. hyodysenteriae genome (Fig. 3). In B. hyodysenteriae B78\textsuperscript{T} Bh100 hybridised to an approximately 800-kb Ecl\textsuperscript{XI} fragment. In order to determine whether fragments Ecl\textsuperscript{XI}-A or Ecl\textsuperscript{XI}-B [12] were hybridised, PFGE with improved separation of larger DNA fragments was performed; hybridisation of Bh100 to fragment Ecl\textsuperscript{XI}-A was observed and confirmed by hybridising the same blot with the \textit{nox} gene previously mapped to this fragment [12]. Fragment Bh400 hybridised to the 320-kb Sau3A-B and 420-kb Ecl\textsuperscript{XI}-E fragment of B. hyodysenteriae B78\textsuperscript{T} [12]. These results show that the fragments Bh100 and Bh400 are not part of a single locus on the B. hyodysenteriae genome.

3.3. Characterisation of the B. hyodysenteriae-specific gene encompassing Bh100

Analyses of the RDA fragments Bh100 and Bh400 had shown that they were both part of a continuous ORF. However, most likely due to their insufficient length, data-

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Fig. 2. Southern blots of \textit{Brachyspira} spp. reference strains and field isolates using fragments Bh100, Bh400, and a \textit{nox} gene-derived DNA fragment as probes. The chromosomal DNA is digested with Eco\textit{RV}. A: B. hyodysenteriae B204 and 20 B. hyodysenteriae field isolates (lanes 2–21) hybridised with Bh100 (top) and Bh400 (bottom). B: B. hyodysenteriae B204 (lane 1), B. murdochii C301 (lane 2), B. innocens C336 (lane 3), B. intermedia AN26/93 (lane 4), B. pilosicoli P436/78 (lane 5), B. murdochii field isolates (lanes 6–11), B. innocens field isolates (lanes 12–16), B. intermedia field isolate (lane 17), B. pilosicoli field isolates (lanes 18–21) hybridised with Bh100 (top), Bh400 (middle), and a \textit{nox} gene fragment (bottom).
base searches did not reveal any significant homology to known proteins. Therefore, DNA overlapping the RDA fragments was cloned and analysed by nucleotide sequencing. In order to investigate fragment Bh100, two DNA fragments (Bh106 and Bh104) having a total length of 6.5 kb were cloned (Fig. 4A) as no *E. coli* transformant containing the 4.5-kb *Hind*III could be isolated even in repeated experiments. This led to the hypothesis that the

![Fig. 3. Localisation of the RDA fragments Bh100 and Bh400 on B. hyodysenteriae B78T chromosome. A: Ethidium bromide-stained gel (left) and Southern blots using Bh100 (middle) and Bh400 (right) as probes. The lanes contain Lambda concatemers (M), B. hyodysenteriae strains B204 and B78T digested with *Sal*I (lanes a, b) and with *Eag*I (lanes c, d). The numbers to the left indicate the size of the marker (in kb); the numbers to the right indicate the size of the relevant fragments hybridising in the digests of B. hyodysenteriae B78T DNA (in kb). B: Physical map of the B. hyodysenteriae B78T chromosome [12]. The arrows indicate the *Eco*XI and the *Sal*I fragments hybridised by Bh100 and Bh400, respectively.

![Fig. 4. Physical map of chromosomal DNA encompassing the RDA fragments Bh100 (A) and Bh400 (B). The solid arrows indicate the size and relative position of the B. hyodysenteriae-specific ORFs; the grey boxes indicate the position of the RDA fragments, and the open boxes indicate the large fragments cloned and sequenced. The numbers in parentheses indicate the relative position in bp.](https://academic.oup.com/femsle/article-abstract/210/2/173/487872)

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entire ORF containing Bh100 might be lethal to transformed E. coli, and the alternative strategy described was taken. Sequencing analysis (GenBank accession no. AY074796) revealed that the RDA fragment Bh100 was part of an ORF of 2130 bp in length. The predicted amino acid sequence was subjected to a database search using BLASTN and FASTA. No homology to any bacterial protein was found, and the limited homology of 20.1% over 244 amino acids to the sensory transduction histidine kinase gene of Dictyostelium discoideum also did not give a hint towards the function of this ORF (Table 2). Therefore, a targeted mutagenesis approach [10] will have to be taken in order to investigate the function of this gene in B. hyodysenteriae.

3.4. Characterisation of the B. hyodysenteriae-specific gene encompassing Bh400

In order to investigate fragment Bh400, a 2.5-kb HindIII fragment (Bh402) was cloned (Fig. 4B). Sequence analysis (GenBank accession no. AY072733) revealed two ORFs, ORF1 (1392 bp) and ORF2 (854 bp), with significant homologies over their total length to the fructose-specific phosphotransferase-system (PTS) and to the fructose-biphosphate aldolase of Bacillus subtilis, respectively (Table 2). As ORF1 did not contain fragment Bh400 (used as a probe to investigate B. hyodysenteriae specificity) Southern hybridisation analysis was repeated using the NsiI–BorGI fragment from within ORF1 as a probe. The results were identical to those obtained with Bh400 (data not shown) showing that the entire PTS homologue is B. hyodysenteriae-specific. PTSs in general have three functions, (i) the transport and phosphorylation of carbohydrates, (ii) positive chemotaxis towards these carbohydrates, and (iii) the regulation of other metabolic pathways [23]. In relation to virulence it is interesting to note that chemotaxis of B. hyodysenteriae towards intestinal mucus is considered to be a virulence factor [24], and no genes involved in this behaviour of B. hyodysenteriae have been investigated to date. Therefore, future work will need to investigate the chemotaxis of B. hyodysenteriae towards fructose and, by constructing isogenic mutants, the role of this putative B. hyodysenteriae-specific PTS operon in virulence.

Acknowledgements

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References