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Identification of novel potential virulence associated factors in *Haemophilus parasuis*

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ABSTRACT

Haemophilus (H.) parasuis is best known as the cause of Glässer's disease, a potentially fatal polyserositis in pigs. The mechanisms underlying virulence differences on the molecular level are largely unknown to date. We have compared the serotype 5 (causes polyserositis) and 11 (described as avirulent) reference strains by modified representational difference analysis, and identified five potentially virulence associated factors present in the invasive serotype 5 strain, but not in the avirulent serotype 11 strain. Among these, a putative hemolysin operon, *hhdBA*, was identified, which is also present in half of the serotype reference strains described as virulent, but not in those reference strains that were reported to cause no disease in animal infection experiments. The presence of all identified genes was investigated in serotype reference strains as well as in 26 field isolates from clinically ill pigs. Determining the presence of these genes may be useful in *H. parasuis* diagnostics to judge a strain's potential to cause disease.

KEYWORDS

Haemophilus parasuis, virulence, hemolysin, toxin, iron transport

INTRODUCTION

Haemophilus parasuis, a Gram-negative NAD dependent rod of the *Pasteurellaceae* family, has gained considerable importance in recent years. Originally recognized as the causative agent of a fibrinous polyserositis termed Glässer's disease, *H. parasuis* disease is now known to cause a variety of clinical pictures especially in herds of high health status. In addition to polyserositis, the organism can cause meningitis, arthritis, pneumonia, or it can behave like a commensal of the respiratory tract and cause no disease at all (Nielsen, 1993). Fifteen serotypes have been recognized so far, and the pathogenic potential of the serotype reference strains has been determined in animal experiments using SPF piglets (Kielstein and Rapp-Gabrielson, 1992). Unfortunately, there is high variation concerning virulence not just between serotypes, but also between different strains of the same serotype (Oliveira and Pijoan, 2004). In addition, many strains are non-typeable by the current serotyping technique (Turni and Blackall, 2005).

Very few virulence associated factors have been identified in *H. parasuis* to date, namely neuraminidase activity (Lichtensteiger and Vimr, 1997), transferrin binding proteins (Charland et al., 1995), and fimbriae (Munch et al., 1992). However, while no gene has been identified for neuraminidase so far, fimbriae and transferrin binding protein genes are likely to be present in all strains of *H. parasuis* (Metcalf and MacInnes, 2007), which precludes an uncomplicated use of these genes for the prediction of the virulence potential of a given strain. Distinct membrane protein patterns have been observed to be correlated with pathogenicity, however, these proteins have not been identified (Oliveira and Pijoan, 2004). A recent attempt to identify virulence factors of *H. parasuis* employed a differential display technique to identify genes expressed upon iron restriction and during growth in cerebrospinal fluid. Several genes were identified that could be relevant or even required for virulence, but the results suggest that they are present in all serotypes and that differences in virulence resulting from these genes would likely be due to differences in their expression. One such

exampe could be a haloacid hydrogenase which was found to be upregulated in virulent reference strains (Metcalf and MacInnes, 2007).

In order to improve the examination of *H. parasuis* strains for their potential to cause disease, it would be desirable to identify genes that are uniquely present in virulent strains. In the present study, we compared two serotype reference strains of *H. parasuis* which are known to cause different clinical pictures in order to identify such genes.

MATERIALS AND METHODS

Primers.

The primers used in this work are listed in Table 1.

Media and growth conditions.

Escherichia coli strains were cultured in Luria-Bertani (LB) medium supplemented with the appropriate antibiotics (ampicillin, 100 µg/ml; kanamycin, 50 µg/ml); *H. parasuis* strains were cultured at 37°C in BHI medium (Difco GmbH, Augsburg, Germany), chocolate agar, PPLO agar (Difco GmbH, Augsburg, Germany) supplemented with NAD (10 µg/ml, E. Merck AG, Darmstadt, Germany), Columbia Sheep Blood agar (CSB, Difco GmbH, Augsburg, Germany) or blood agar containing 7% porcine blood and 10 µg/ml NAD, using a *Staphylococcus aureus* nurse strain on plates without added NAD.

Manipulation of nucleic acids and proteins.

Nucleic acid modifying enzymes were purchased from New England Biolabs (Bad Schwalbach, Germany) and used according to the manufacturer's instructions. *Taq* polymerase was purchased from Invitrogen (Karlsruhe, Germany) and Promega (Mannheim, Germany).

Modified Representational Difference Analysis (RDA).

A subtractive hybridization protocol based on RDA (Lisitsyn et al., 1993) was employed as described previously (Strommenger et al., 2001). Briefly, DNA from *H. parasuis* serotype reference strains 5 and 11 (Kielstein and Rapp-Gabrielson, 1992) were digested with DpnI

and DpnII, followed by the ligation of double stranded oligonucleotide adaptors RBgl12/RBgl24 to serotype 5 DNA fragments only. Then, 0.2 µg of serotype 5 DNA (tester) were hybridized to 20 µg of serotype 11 DNA (driver) at 67 °C for 20 hours, followed by PCR using primer RBgl24 as described for RDA (Lisitsyn et al., 1993). Resulting PCR products were cloned using the StrataClone® PCR cloning kit (Stratagene, USA). Inserts were then amplified from these clones, run on an agarose gel, subjected to Southern blotting, and hybridized to P^{32} labeled *H. parasuis* tester and driver DNA, respectively. Fragments that gave stronger signals with serotype 5 DNA were sequenced (SeqLab GmbH, Göttingen, Germany). To eliminate false positives, the nucleotide sequences were then used to generate primers for PCR. Sequences were then confirmed to be present in serotype 5, but absent from serotype 11 by PCR as well as Southern blotting using the generated PCR products as P^{32} labeled probes on genomic serotype 5 and 11 DNA.

RT-PCR

Aerobic *H. parasuis* cultures were grown in a shaking incubator to OD_{600} = 0.4.

For RT-PCR, RNA was prepared from 5 ml of liquid culture using RNEasy mini columns (Qiagen, Germany) and DNAse treated (Turbo DNAse, Applied Biosystems, Germany) according to the manufacturers' instructions. Reverse transcription was carried out by setting up a mastermix containing 5 µg of RNA with 50 pmol of random hexamer primers, then splitting up the sample into two equal portions, to one of which reverse transcriptase (SuperScriptII, Invitrogen, Germany) was added. After 60 minutes at 37°C, cDNA template and control samples were diluted 1:100 with ddH_2O and 5 µl served as template for PCR (94°C 3 min, [94°C 30 sec, 55°C 1 min, 72°C 1:30 sec] x 32, 72 °C 10 min). RT-PCR experiments were performed in triplicate.

RESULTS

Identification of serotype 5-specific *H. parasuis* genes.

Five genes were identified that are present in the *H. parasuis* serotype 5 but not in the serotype 11 reference strain. They were found in the incomplete genomic sequence for *H. parasuis* available at NCBI (http://www.ncbi.nlm.nih.gov): putative iron transporter *cirA* (NZ_ABKM01000010, positions 26233-25742), two components of a putative hemolysin/ export system *hhdA*/*hhdB* (ZP_02479317, ZP_02479316) which are organized successively as *hhdBA*, and two putative phage related genes which were discovered in a single sequenced RDA clone (ZP_02477853, ZP_02477854). On the protein level, *H. parasuis cirA* is 76 % identical and 88% similar to CirA of "*Mannheimia succiniproducens*" (YP_088507). Genes *hhdB* and *hhdA* are 40% identical and 62 % similar to an uncharacterized hemolysin in *H. ducreyi* (NP_873758, NP_873759), and they also show homology to similarly organized hemolysins in *Serratia* and *Yersinia* species. The two possibly phage related genes are annotated as "hypothetical proteins" in the *H. parasuis* genome, however, when compared to other species, ZP_02477853 is 83% identical and 89% similar to a phage accociated restriction endonuclease from *A. pleuropneumoniae* (YP_001968611), and ZP_02477854 is 81% identical, 91% similar to a putative phage DNA packaging protein from *A. pleuropneumoniae* (YP_001968612). Since both genes were originally obtained from a single RDA clone, the sequence obtained from this clone was used for primer generation so that both genes would be picked up in a single PCR reaction. All five genes were confirmed to be present in the serotype 5, but absent in the serotype 11 reference strain by PCR (Fig. 1) and Southern blot (not shown).

Presence of serotype 5 strain *Nagasaki* specific genes in other *H. parasuis* serotype reference strains and field isolates.

PCR analysis was used to test for the presence of the identified genes in all 15 serotype reference strains (Kielstein and Rapp-Gabrielson, 1992, table 2) and in 26 field isolates from

independent cases of clinically ill pigs in northern Germany (table 3); in addition, Southern blot analysis was performed on the serotype reference strains to confirm PCR results (not shown). Using primer pairs MP_A1/2, MP_B1/ 2 , and MP_CirA1/2, *hhdA*, *hhdB* and *cirA* were shown to be present in 3 of 5 highly virulent serotype reference strains, and in 2 of 5 strains that caused polyserositis but not fatal disease. *hhdA* and *hhdB* were absent from 5 serotype reference strains that had been shown to be avirulent in experimental infection of SPF pigs (3, 6, 7, 9, 11, Kielstein et al., 1991). *H. parasuis cirA* was present in the avirulent serotype 3 reference strain, but not in the other strains described as avirulent (table 2). The phage related genes were found in serotype reference strains 2, 5, 12.

We hypothesized that strains isolated from systemic sites were more likely to represent virulent strains than strains isolated from the lungs which might represent a co-infecting strain rather than the one responsible for disease. Therefore, 26 field isolates (confirmed by PCR according to Oliveira et al., 2001, not serotyped) from clinically ill pigs were divided into two groups: 1) Ten invasive strains which were isolated from the meninges, joints, pericardium, or thoracic or abdominal cavities; 2) sixteen strains isolated from pneumonic lungs at high cfu counts with signs of invasive disease not necessarily present. Half of the invasive field isolates possess *hhdA* and *cirA*, but only 2 of 10 strains possess the phage related genes. Except for two systemic strains which were only positive for *hhdA*, *hhdB* and *hhdA* were always detected together in the same strain. In the lung-derived isolates, 6 of 16 strains carried *hhdBA* and the phage terminase, whereas *cirA* was found in 14 of 16 strains isolated from pig lungs (table 3). Notably, across all the strains tested in this study, those carrying *hhdA* also carried *cirA.* To test for the presence of different alleles of *hhdB* and *cirA* genes in the reference strains that tested negative in PCR and Southern blot, primer pairs oHHD503/502 and oCirA101/102, were used. While the *cirA* primers yielded the same results as previous analyses, we obtained *hhdB* products for serotypes 2, and 7-11, which had previously been negative. The nucleotide sequence of these PCR products was identical to serotype 5 *hhdB*.

Transcription.

In order to demonstrate that the five genes were expressed in *H. parasuis* serotype 5 strain "Nagasaki", RNA was prepared and subjected to reverse transcription and PCR. Transcripts were found for all five genes and confirmed by nucleotide sequencing (Fig. 2).

DISCUSSION

The ability of some strains of *H. parasuis* to colonize the porcine respiratory tract without causing disease poses a major difficulty for *H. parasuis* diagnostics. The variability of the strains of a given serotype together with the frequent occurrence of nontypeable isolates limits the usefulness of serotyping to assess a particular strain's potential for virulence (Blackall et al., 1997). In order to properly characterize a strain, the presence or absence of virulence associated factors needs to be investigated. In the study presented here, we have identified several previously undescribed potential virulence-accociated genes and investigated their distribution between serotype reference strains and 26 field isolates. All five genes are expressed by *H. parasuis* serotype 5 reference strain *Nagasaki* in vitro.

We identified a novel potential hemolysin/export system, *hhdBA*. In *H. ducreyi*, *hhdB* is expressed during infection in human volunteers, but does not seem to be crucial for virulence (Throm and Spinola, 2001). However, it has been shown to be immunogenic (Dutro et al., 1999). *H. parasuis* is nonhemolytic on CSB and pig blood agar, which indicates that *hhdBA* is either functional only in the host or it might be a toxin with a different function, perhaps active at sublytical concentrations. It appears that different alleles of *hhdBA* exist in the *H. parasuis* species, as demonstrated by differing PCR and Southern blot results. The currently available data suggests that *hhdBA* of different serotypes contains conserved as well as variable regions, similar to the situation with transferrin receptor subunit *tbpB* in *A. pleuropneumoniae* (Strutzberg et al., 1995). The frequent occurrence of serotype 5 *hhdBA* in invasive strains is notable, however, about half of the invasive field isolates we investigated

do not possess the same *hhdBA* as serotype 5. In addition, there was no marked difference between systemic and lung-derived isolated concerning the presence of *hhdBA*. Possible explanations could be that *hhdBA* does not play a pivotal role for invasion or virulence, or that its function is compensated by different genes in the other invasive strains. Since the prediction of *hhdBA* function relies on in silico analysis and similarity to known proteins, *hhdBA* needs to be further investigated before its exact role as a potential hemolysin/toxin in *H. parasuis* pathogenesis can be determined. Genetic variation of *hhdBA* could be useful for typing purposes.

The *cirA* gene appears to be widely distributed among *H. parasuis* strains. It is the only gene present in almost all field strains. In *E. coli*, *cirA* is iron-regulated and postulated to be in involved in enterobactin transport, and can also serve as a receptor for antibiotics like colistin and catechol-substituted cephalosporins (Nikaido and Rosenberg, 1990). Interestingly, all *hhd* positive *H. parasuis* strains were also positive for *cirA*. Its role in *H. parasuis* remains to be elucidated; however, iron-regulated proteins have been shown to be involved in or even required for virulence in many bacteria including members of *Pasteurellaceae* and *Neisseriaceae* (Cornelissen et al., 1998, Baltes et al., 2002).

The two phage related genes are only present in a small number of *H. parasuis* strains. As shown by RT-PCR analysis, the two genes identified from the RDA clone are transcribed on a single mRNA. Without any further data on a complete phage, nothing can be said about its possible contribution to virulence. However, phages are implicated in virulence in many organisms due to their ability to carry and transmit virulence-associated genes, such as toxins, famous examples being the cholera toxin of *Vibrio cholerae* and the Shiga toxin of *E.coli* (Waldor and Friedman, 2005).

Our study is the first report of a putative hemolysin in *H. parasuis*. It must be noted that the observation that a certain allele of *hhdBA* appears to be associated with an ability to cause disease is currently limited to the serotype reference strains which were the only strains in our

study for which virulence has been individually investigated in animal experiments (Wiegand et al., 1997).

Only about half of the 26 *H. parasuis* field isolates from clinically ill pigs carried the genes we identified, and the genes cannot be used to discriminate between pneumonic and systemic strains. Obviously, taking into account the absence of these genes in some virulent reference strains, the absence of these genes cannot be interpreted as an indicator of avirulence for any given field isolate. However, assuming the genes we identified are indeed associated with virulence, it is likely that other virulent strains will either possess different alleles of these genes, or entirely different factors that remain to be identified. Alternatively, since more than one strain can colonize the same animal (Smart et al., 1988), it is possible that a particular clinical isolate is not the one responsible for the outbreak of disease. Further work is required to determine the distribution of these genes among strains of a single serotype, as well as their contribution to virulence. In comparison to the study by Metcalf and MacInnes (Metcalf and MacInnes, 2007), our study focused on the presence or absence of genes rather than differences in expression levels. The authors found a correlation between elevated haloacid dehydrogenase expression with virulence for a subset of the reference strains, but did not investigate all reference strains, or additional field strains, and the role of haloacid dehydrogenases in virulence has not been fully investigated in *H. parasuis* or in other organisms. Perhaps a multifaceted approach, looking at the presence, variability and expression level of genes will be able to predict the pathogenic potential of *H. parasuis* strains more reliably when more data is available.

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Table 1: Primers used in this study

primers	description	source
RBgl12 RBgl24	5' GATCTGCGGTGA 3' 5' AGCACTCTCCAGCCTCTCACCGCA 3' Primer pair to generate RDA adaptor	(Lisitsyn et al., 1993)
HPS-forward HPS-reverse	5' GTGATGAGGAAGGGTGGTGT 3' 5' GGCTTCGTCACCCTCTGT 3' Primer pair for amplification of 16 S rRNA gene fragment, product size 821 bp	(Oliveira et al., 2001)
MP_A1 MP_A2	5' GGTTCTAGTTCACAAACAGCCAATAC 3' 5' GATATTTACCCCTGCCTTCATTGTATC 3' Primer pair for amplification of *hhdA* gene fragment, product size 964 bp	this work
MP_B1 MP_B2	5' ATCTTGCCCTGATTAGAGAGTAGGAGT 3' 5' GTGAATATAGCCCTTATCCAAATAGGC 3' Primer pair for amplification of *hhdB* gene fragment, product size 557 bp	this work
oHhdB1 oHhdB2	5'-CTTACGCCTTGTTTGATCTG-3' 5'-TATGTTGCATGGGTGCTA-3' product size 348 bp	this work
oHHD503 oHHD502	5'-AGCGAGTATCATCGGTGGTC-3' 5'-CTTGGCTGACAATTCAGCTT-3' product size 509 bp	this work
MP_CirA1 MP_CirA2	5' GTATGCAGAATAAAGCCCTGCTAAAC 3' 5' AAAGAGCCGAGAAATATCGTAGATGTG 3'	this work

	Primer pair for amplification of *cirA* gene fragment in RT-PCR, product size 161 bp	
MP_CirA1 MP_CirA4	5' GTATGCAGAATAAAGCCCTGCTAAAC 3' 5' CTGTAAAGCAATGCAATTACCGTAGTG 3' Primer pair for amplification of *cirA* gene fragment, product size 215 bp	this work
oCIR101 oCIR102	5' CGCACACGGATCAGAGAGTA 3' 5' GGTGGTAAACCGCTTGATCT 3' Primer pair for amplification of *cirA* gene fragment, product size 1593 bp	this work
oPhage13-1 oPhage13-2	5' GCTTGCGGGTAATCTGTTGT 3' 5' AGAATCAACCTCAGCCGAAA 3' Primer pair for amplification of phage related genes (upstream primer in ZP_02477853, downstream primer in, ZP_02477854), product size 301 bp	this work

Table 2: PCR of *H. parasuis* serotype reference strains, sorted by virulence according to Kielstein and Rapp-Gabrielson, 1992, presence of identified genes

	serotype reference strain														
gene	**death within 4 days**					**polyserositis, but no death within 4 days**					**no clinical symptoms**				
	1	5	10	12	13	14	2	4	8	15	3	6	7	9	11
hhdA	-	+	-	+	+	+	-	-	-	+	-	-	-	-	-
hhdB	-	+	-	+	+	+	-	-	-	+	-	-	-	-	-
cirA	-	+	-	+	+	+	-	-	-	+	+	-	-	-	-
phage	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-

1 Table 3: PCR analysis of *H. parasuis* field isolates, presence of identified genes

	in strains isolated from systemic locations	in strains isolated from lungs
hhdA	5/10	6/16
hhdB	3/10	6/16
cirA	5/10	14/16
phage	2/10	6/16

2

FIGURE LEGENDS

Fig. 1: PCR analysis of identified genes in *H. parasuis* serotype reference strains 1-15. +: *H. parasuis* serotype 5 "Nagasaki" as positive control, -: no DNA template

Fig. 2: Transcription of identified genes in *H. parasuis* serotype 5 reference strain "Nagasaki", assessed by reverse transcriptase PCR. 1: *hhdA*, 2: *hhdB*, MP_B1/2. 3: *cirA*, 4: phage related genes, 5: *H. parasuis* 16S RNA (Oliveira et al., 2001). +RT: samples with reverse transcriptase, - RT: samples without reverse transcriptase. DNA: positive controls containing genomic DNA template. -: no DNA template

Figure 1

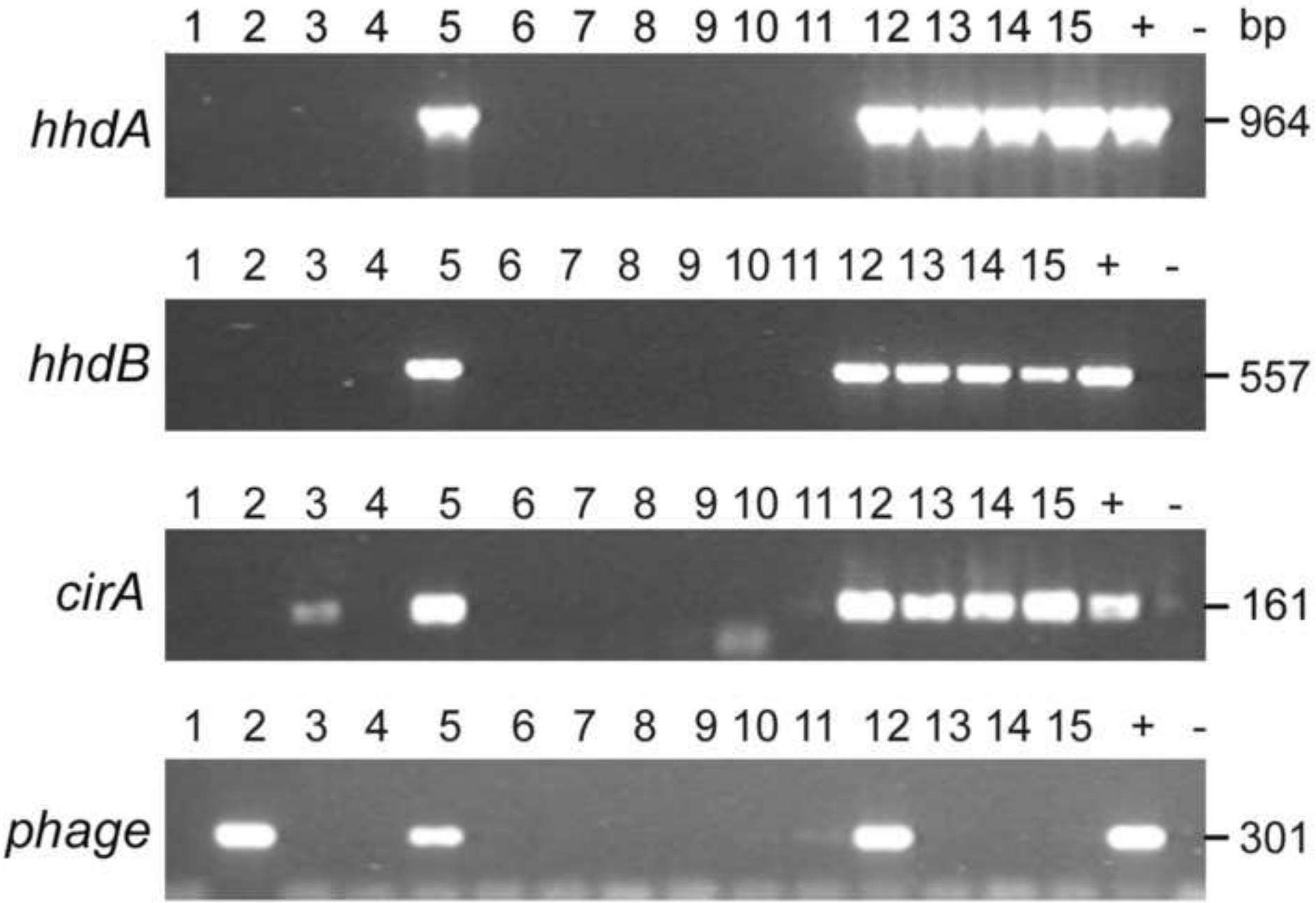
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 + - bp
hhdA
964
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 + -
hhdB
557
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 + -
cirA
161
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 + -
phage
301

Figure 2