Functional characterization of the BvgAS two-component system of *Bordetella holmesii*

Gabrielle Gerlach, Simone Janzen, Dagmar Beier and Roy Gross

Theodor-Boveri-Institut für Biowissenschaften, Lehrstuhl für Mikrobiologie, Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany

The BvgAS two-component system is the master regulator of virulence gene expression in the mammalian pathogens *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. This paper reports the partial cloning and characterization of the bvgAS loci of the ‘new’ *Bordetella* species *Bordetella holmesii*, *Bordetella trematum* and *Bordetella hinzii*, which are increasingly recognized as opportunistic pathogens in humans. It is demonstrated that the cytoplasmic signalling domains of the BvgS histidine kinases of *B. pertussis* and *B. holmesii* are functionally interchangeable, while signal perception by the two sensor proteins seems to be different. Furthermore, it is shown that, despite the high similarity of the BvgA proteins of *B. pertussis* and *B. holmesii*, promoter recognition by the response regulator proteins differs substantially in these organisms.

INTRODUCTION

The members of the genus *Bordetella* form a group of closely related organisms. With the exception of *Bordetella petrii*, which was recently isolated from river sediment (von Wintzingerode et al., 2001), *Bordetella* species so far have been found exclusively in close association with host organisms (Cotter & DiRita, 2000; Gerlach et al., 2001). The so-called ‘classical’ *Bordetella* species include *Bordetella pertussis*, *Bordetella parapertussis*, *Bordetella bronchiseptica* and *Bordetella avium*. The human pathogens *B. pertussis* and *B. parapertussis* are the causative agents of whooping cough and a milder pertussis-like disease, respectively. *B. bronchiseptica* causes respiratory infections in a wide range of warm-blooded animals and *B. avium* is the aetiological agent of bordetellosis, an upper respiratory tract disease of young poultry and other birds (Skeels & Arp, 1988). The ‘classical’ *Bordetella* species are quite well characterized and, with the exception of *B. avium*, whose genome sequence is in the finishing phase, their genome sequences have already been published (Parkhill et al., 2003).

Within the past few years, four new species have been added to the genus *Bordetella*. *Bordetella holmesii* can cause disease in man and has been isolated from blood cultures of patients with underlying disease and from patients with whooping cough-like symptoms (Weyant et al., 1995; Tang et al., 1998; Yih et al., 1999; Mazengia et al., 2000; Shepard et al., 2004).

*Bordetella hinzii* appears to be a commensal of birds, although it has been isolated occasionally also from the blood of immunocompromised patients (Cookson et al., 1994; Kattar et al., 2000). *Bordetella trematum* was isolated from patients suffering ear or wound infections, but was not found in the respiratory tract (Vandamme et al., 1996); at present its pathogenic potential is not known. Finally, *B. petrii* was the first member of the genus to be isolated from the environment (von Wintzingerode et al., 2001). Little is known about the biology and the pathogenic potential of these ‘new’ *Bordetella* species, although *B. holmesii* is increasingly recognized as being associated with human disease (Yih et al., 1999).

During the course of the development of new vaccines against whooping cough, much has been learnt about the pathogenicity and the virulence-associated factors of *B. pertussis*, *B. bronchiseptica* and *B. parapertussis*. Interestingly, these three species share several virulence-relevant factors (Hewlett & Cowell, 1989; Weiss, 1992; Parton, 1999), including adhesion or colonization factors, such as the filamentous haemagglutinin, fimbriae of several serotypes and several autotransporter proteins. Moreover, these bacteria produce highly related toxic factors such as the trachael cytotoxin, the dermonecrotic toxin and the adenylate cyclase toxin. Pertussis toxin is expressed exclusively by *B. pertussis*, although the ptx genes are also present in *B. parapertussis* and *B. bronchiseptica* (Gross & Rappuoli, 1988). It is well known that the three closely related *Bordetella* species *B. pertussis*, *B. bronchiseptica* and *B. parapertussis*, which are also referred to as members of the *B. bronchiseptica* cluster (Gerlach et al., 2001), coordinately regulate the expression of these virulence factors via a

Abbreviations: Cya, adenylate cyclase toxin; FhaB, filamentous haemagglutinin; GFP, green fluorescent protein.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AJ748854, AJ748855 and AJ48856.
highly conserved two-component system encoded by the bvgAS locus (Weiss et al., 1983; Arico et al., 1989; Cotter & DiRita, 2000; Bock & Gross, 2001).

BvgAS mediates the transition between the virulent and the avirulent phenotype by two phenomena called antigenic modulation and phase variation. Antigenic modulation describes a reversible on-and-off switch in the expression of pathogenicity factors in response to environmental signals, such as temperature, magnesium sulphate or nicotinic acid, and is caused by changes in the phosphorylation state of the BvgAS two-component system (Lacey, 1960; Uhl & Miller, 1996; Zu et al., 1996; Perraud et al., 1998). In contrast, phase variation, which occurs in vitro at a strain-dependent frequency of 10^{-2}–10^{-6}, is characterized by the irreversible loss of expression of the virulence-associated genes irrespective of the growth conditions (Leslie & Gardner, 1931; Peppler, 1982; Peppler & Schrumpf, 1984). It has been shown in a B. pertussis isolate that phase variation was due to a frameshift mutation in the bvgS gene (Stibitz et al., 1989), while in B. bronchiseptica, phase variation is frequently caused by small deletions within the bvgS gene (Monack et al., 1989).

In contrast to the ‘classical’ species, virtually nothing is known about virulence-relevant features of the ‘new’ Bordetella species. Since the BvgAS system is the master regulator of virulence in the ‘classical’ species, we characterized the orthologous genes from the ‘new’ species in order to use the BvgAS system as a starting point for the identification of possible virulence factors in these species. In the present paper we describe the properties of the BvgAS systems of the new species, with the main emphasis on the human pathogen B. holmesii.

METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. B. holmesii strains were grown in brain heart infusion (BHI; Difco) or on Bordet–Gengou (BG) agar plates supplemented with 20% horse blood (Bordet & Gengou, 1909). B. pertussis strains were grown in modified Stainer–Scholte medium (Stainer & Scholte, 1970; Aoyama et al., 1986) or on BG agar plates. When required, antibiotics were added to the following final concentrations: streptomycin, 100 μg ml^{-1}; kanamycin, 25 μg ml^{-1}; tetracycline, 12.5 μg ml^{-1}; ampicillin, 100 μg ml^{-1}; and chloramphenicol, 15 μg ml^{-1}. Bacterial conjugations were performed as described previously (Gross & Rappuoli, 1988), using Escherichia coli SM10 as the donor strain (Simon et al., 1983). Protein lysates were prepared from bacteria grown on BG agar plates for 72 h at 37 °C which were suspended in saline at a cell density of 2.4 × 10^{9} c.f.u. ml^{-1}.

General techniques. DNA manipulation, cloning procedures and SDS-PAGE were carried out according to standard procedures (Sambrook & Russell, 2000). PCR amplifications were performed with a model Progene thermocycler (Techne) by using Deep Vent DNA polymerase (New England Biolabs) or Taq polymerase (Qbiogene). All cloned PCR products were subjected to automated sequencing (Big Dye kit; Perkin-Elmer) to ensure proper amplification. Immunoblot analysis was performed using a semidry blotting procedure as described by Towbin et al. (1979). Membrane-immobilized filamentous haemagglutinin (FhaB) and green fluorescent protein (GFP) were detected using goat FhaB and rabbit GFP antisera (Invitrogen), respectively.

Characterization of the bvgAS loci of B. holmesii, B. trematum and B. hinzii. PCR reactions with degenerate oligonucleotide primers were performed essentially as described by Morel-Deville et al. (1997) using chromosomal DNA of B. holmesii G7702, B. trematum and B. hinzii as templates. The degenerate primers were deduced from highly conserved regions of the receiver domain and from the output domain of BvgA, from B. pertussis. Plasmids and oligonucleotides used in this study are listed in Table 1 and Table 2, respectively.

The PCR products were cloned into pGEM-T vector DNA, giving rise to plasmids pGEM-T-deg1BH, pGEM-T-deg1BHZ, pGEM-T-deg2BH and pGEM-T-deg2BT, and sequenced. Sequence analysis demonstrated that the cloned DNA fragments were indeed derived from the bvgA orthologues of the ‘new’ Bordetella species. Interestingly, primer deg2-reverse turned out to have annealed to a sequence motif within bvgS, which is located further upstream than the motif from which the degenerate primer sequence was derived, therefore yielding PCR fragments of unexpected length.

Partial genomic libraries of B. holmesii were screened using PCR fragment ‘deg1BH’ as probe. The DNA inserts of positive clones were subsequently sequenced to assemble the complete nucleotide sequence of bvgBH including its flanking DNA regions. The nucleotide sequences of ‘deg1BH’ and ‘deg2BT’ were extended by a combination of different techniques including inverse PCR, genome walking [using the Universal Genome Walker Kit (Clontech)] and the screening of partial genomic libraries of the respective organisms.

Cloning of the bvgASBH locus of B. holmesii ATCC 51541 (bvgASBH ATCC 51541) and B. holmesii G7702 (bvgASBH G7702). In order to construct plasmids pSL-bvgASBH G7702 and pSL-bvgASBH ATCC 51541 the 4.7 kbp DNA region comprising the orfx-bvgASBH intergenic region and the entire bvgASBH locus, from B. holmesii G7702 and ATCC 51541, was assembled by stepwise cloning from three individual PCR fragments (bvgASBH-F1, bvgASBH-F2 and bvgASBH-F3) amplified from chromosomal DNA of the corresponding B. holmesii strain. The following steps were repeated for both B. holmesii strains. PCR fragment bvgASBH-F1, which includes the orfx-bvgASBH intergenic region and extends to the SphI site of the bvgASBH gene, was synthesized using primer pair bvgASBH-F1-SphI/bvgASBH-F1-SphI. PCR using primer pairs bvgASBH-F2-SphI/bvgASBH-F2-KpnI and bvgASBH-F3-KpnI/bvgASBH-F3-EcoRI, respectively, yielded DNA fragment bvgASBH-F2, containing the DNA segment located between the SphI and KpnI site of the bvgASBH gene, and PCR fragment bvgASBH-F3, containing the sequence flanked by the KpnI site and the stop codon of the bvgASBH gene. Fragments bvgASBH-F1 and bvgASBH-F2 were first ligated into...
<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Relevant feature(s)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. holmesii</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 51541</td>
<td>Clinical isolate</td>
<td>Weyant et al. (1995)</td>
</tr>
<tr>
<td>No1</td>
<td>Clinical isolate</td>
<td>Njamkepo et al. (2000)</td>
</tr>
<tr>
<td>G8341</td>
<td>Clinical isolate</td>
<td>Weyant et al. (1995)</td>
</tr>
<tr>
<td>G7702</td>
<td>Clinical isolate</td>
<td>Weyant et al. (1995)</td>
</tr>
<tr>
<td>G7702/bvgABH::kan</td>
<td>G7702 with a kanamycin-resistance cassette disrupting the <em>bvgABH</em> gene</td>
<td>This study</td>
</tr>
<tr>
<td><strong>B. pertussis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>347</td>
<td>Derivative of TI, <em>bvgS</em>::Tn5</td>
<td>Weiss et al. (1983)</td>
</tr>
<tr>
<td><strong>B. hinzii</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 51783</td>
<td>Isolate from the trachea of a chicken</td>
<td>Vandamme et al. (1995)</td>
</tr>
<tr>
<td><strong>B. trematum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCUG32381</td>
<td>Human isolate</td>
<td>Vandamme et al. (1996)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5x</td>
<td>Strain used for high-efficiency transformation</td>
<td>Gibco</td>
</tr>
<tr>
<td>M15</td>
<td>Strain used for overproducing His$<em>r$-Bvg$</em>{ABH}$ and His$<em>r$-Bvg$</em>{SN}$</td>
<td>Qiagen</td>
</tr>
<tr>
<td>SM10</td>
<td>Mobilizing strain</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-T</td>
<td>Vector for cloning of PCR products containing 3’-T overhangs</td>
<td>Promega</td>
</tr>
<tr>
<td>pUC18</td>
<td>High-copy-number cloning vector</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>pRK415</td>
<td>Derivate of pRK404, broad-host-range cloning vector</td>
<td>Keen et al. (1988)</td>
</tr>
<tr>
<td>pSL1180</td>
<td>Cloning vector</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>pMMB208</td>
<td>Broad-host-range expression vector</td>
<td>Morales et al. (1991)</td>
</tr>
<tr>
<td>pSS1129</td>
<td>Used for allelic exchange mutagenesis in <em>Bordetella</em> species</td>
<td>Stibitz &amp; Yang (1991)</td>
</tr>
<tr>
<td>pUC4K</td>
<td>Source of the kanamycin cassette</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>pKEN</td>
<td>Plasmid containing the promoterless <em>gfp</em>-mut2 gene</td>
<td>Cormack et al. (1996)</td>
</tr>
<tr>
<td>pLA57vir</td>
<td>Broad-host-range vector pLAFR2 carrying the entire <em>bvgABSN</em> locus of <em>B. pertussis</em></td>
<td>Arico et al. (1989)</td>
</tr>
<tr>
<td>pProm67</td>
<td>pGem3 carrying a 1250 bp <em>PstI/PstI</em> DNA fragment that includes both promoter regions for the <em>bvg</em> locus and for the <em>fhuB</em> gene of <em>B. pertussis</em>.</td>
<td>Scarlato et al. (1990)</td>
</tr>
<tr>
<td>pCYT-BvgABP</td>
<td>pCYTEXP1 expressing the response regulator Bvg$_{ABP}$ of <em>B. pertussis</em></td>
<td>Perraud et al. (1998)</td>
</tr>
<tr>
<td>pQE30 and pQE31</td>
<td>Expression vectors for N-terminal His-tag cloning</td>
<td>Qiagen</td>
</tr>
<tr>
<td>pQE-BvgSTRO</td>
<td>pQE31 expressing the His$<em>r$-tagged cytoplasmic domains of the histidine kinase Bvg$</em>{SN}$ of <em>B. pertussis</em></td>
<td>Perraud et al. (1998)</td>
</tr>
<tr>
<td>pGEM-T-deg1BH</td>
<td>pGEM-T carrying a 285 bp PCR fragment of <em>B. holmesii</em> G7702 encoding amino acids 7–100 of Bvg$_{ABH}$</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM-T-deg1BH2Z</td>
<td>pGEM-T carrying a 285 bp PCR fragment of <em>B. hinzii</em> encoding amino acids 7–100 of Bvg$_{ABH}$</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM-T-deg2BH</td>
<td>pGEM-T carrying a 237 bp PCR fragment of <em>B. holmesii</em> G7702 encoding amino acids 97–176 of Bvg$_{ABH}$</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM-T-deg2BT</td>
<td>pGEM-T carrying a 237 bp PCR fragment of <em>B. trematum</em> encoding amino acids 97–176 of Bvg$_{ABT}$</td>
<td>This study</td>
</tr>
<tr>
<td>pQE-BvgABH</td>
<td>pQE30 expressing the His$<em>r$-tagged response regulator Bvg$</em>{ABH}$ of <em>B. holmesii</em></td>
<td>This study</td>
</tr>
<tr>
<td>pGEM-T-FP</td>
<td>pGEM-T carrying a 253 bp DNA fragment derived from the upstream region of <em>bvgABH</em></td>
<td>This study</td>
</tr>
<tr>
<td>pSL-bvgABH G7702</td>
<td>pSL1180 carrying the upstream region and the coding region of <em>bvgABH</em> of <em>B. holmesii</em> G7702</td>
<td>This study</td>
</tr>
<tr>
<td>pSL-bvgABH ATCC 51541</td>
<td>pSL1180 carrying the upstream region and the coding region of <em>bvgABH</em> of <em>B. holmesii</em> ATCC 51541</td>
<td>This study</td>
</tr>
<tr>
<td>pRK-bvgABH G7702</td>
<td>pRK415 carrying the upstream region and the coding region of <em>bvgABH</em> of <em>B. holmesii</em> G7702</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 2. Oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’–3’)*</th>
<th>Restriction recognition site</th>
</tr>
</thead>
<tbody>
<tr>
<td>deg1-forward</td>
<td>cgaagctttgathathgaygayca</td>
<td>HindIII</td>
</tr>
<tr>
<td>deg1-reverse</td>
<td>ctcttagaaraanccngcngncncc</td>
<td>XbaI</td>
</tr>
<tr>
<td>deg2-forward</td>
<td>cgaagctttgggngcngcnggnttygt</td>
<td>HindIII</td>
</tr>
<tr>
<td>deg2-reverse</td>
<td>ctcttagaaraanccngcngncncc</td>
<td>XbaI</td>
</tr>
<tr>
<td>bvgASBH1-SpeI</td>
<td>cgcactagtgcaaatgcgtgtagtacgac</td>
<td>SpeI</td>
</tr>
<tr>
<td>bvgASBH1-SphI</td>
<td>acatcggctcggatgtagtgtaaac</td>
<td>SphI</td>
</tr>
<tr>
<td>bvgASBH2-SphI</td>
<td>acatcggctcggatgtagtgtaaac</td>
<td>SphI</td>
</tr>
<tr>
<td>bvgASBH2-F2-KpnI</td>
<td>cgccggtaccctgcggctgtagtacgac</td>
<td>KpnI</td>
</tr>
<tr>
<td>bvgASBH3-F2-KpnI</td>
<td>cgccggtaccctgcggctgtagtacgac</td>
<td>KpnI</td>
</tr>
<tr>
<td>bvgASBH3-F3-EcoRI</td>
<td>cgccggtaccctgcggctgtagtacgac</td>
<td>KpnI</td>
</tr>
<tr>
<td>bvgASBH3-EcoRI</td>
<td>cgccggtaccctgcggctgtagtacgac</td>
<td>KpnI</td>
</tr>
<tr>
<td>bvgASBH3-XbaI</td>
<td>acatctagagcaaatccgtgtagtacgac</td>
<td>KpnI</td>
</tr>
<tr>
<td>bvgASBH3-HindIII</td>
<td>gtaaagcttctgcaagcgggttttt</td>
<td>HindIII</td>
</tr>
<tr>
<td>bvgASBH1-kan^5</td>
<td>gcgcgggacatttcgcaagctgacgagctgac</td>
<td>BamHI</td>
</tr>
<tr>
<td>bvgASBH1-kan^3</td>
<td>gcgcgggacatttcgcaagctgacgagctgac</td>
<td>BamHI</td>
</tr>
<tr>
<td>bvgASBH1-up5</td>
<td>gcgcgggacatttcgcaagctgacgagctgac</td>
<td>BamHI</td>
</tr>
<tr>
<td>bvgASBH1-up3</td>
<td>gcgcgggacatttcgcaagctgacgagctgac</td>
<td>BamHI</td>
</tr>
<tr>
<td>bvgASBH1-BamHI</td>
<td>gcgcgggacatttcgcaagctgacgagctgac</td>
<td>BamHI</td>
</tr>
<tr>
<td>bvgASBH1-KpnI</td>
<td>acatctagagcaaatccgtgtagtacgac</td>
<td>KpnI</td>
</tr>
<tr>
<td>FP1-5</td>
<td>gcgcgggacatttcgcaagctgacgagctgac</td>
<td>BamHI</td>
</tr>
<tr>
<td>FP1-3</td>
<td>acatctagagcaaatccgtgtagtacgac</td>
<td>KpnI</td>
</tr>
<tr>
<td>fhaBup5</td>
<td>gcgcgggacatttcgcaagctgacgagctgac</td>
<td>BamHI</td>
</tr>
<tr>
<td>fhaBup3</td>
<td>gcgcgggacatttcgcaagctgacgagctgac</td>
<td>BamHI</td>
</tr>
<tr>
<td>fhaB-PE</td>
<td>gcgcgggacatttcgcaagctgacgagctgac</td>
<td>BamHI</td>
</tr>
<tr>
<td>bvgASBH1-PE</td>
<td>gcgcgggacatttcgcaagctgacgagctgac</td>
<td>BamHI</td>
</tr>
<tr>
<td>gfp-PE</td>
<td>gcgcgggacatttcgcaagctgacgagctgac</td>
<td>BamHI</td>
</tr>
</tbody>
</table>

*Restriction recognition sequences which were introduced for cloning purposes are underlined.
BvgAS two-component system of B. holmesii

Sple- and KpnI-digested pSK-Bluescript DNA, yielding plasmid pSK-bvgASup/F1/F2. Fragment bvgASup/F1/F2 was excised by Sple and KpnI digestion of pSK-bvgASup/F1/F2 and ligated, together with fragment bvgASup/F3, into Sple- and EcoRI-linearized plasmid pSL1180, generating pSL-bvgASup.

For the construction of plasmids pRK-bvgASup \textit{G7702} and pRK-bvgASup \textit{ATCC 51541}, fragment bvgASup/F1, including the orf-bvgASup intergenic region and extending to the HindIII restriction site of the bvgASup gene, was PCR amplified using primer pair bvgASup-Xbal/bvgASup-HindIII and genomic DNA from the corresponding strain of \textit{B. holmesii}. A DNA fragment, ranging from the HindIII site of the bvgASup gene to the bvgASup stop codon, was excised by HindIII/EcoRI digestion from pSL-bvgASup \textit{G7702} and pSL-bvgASup \textit{ATCC 51541} and subsequently inserted, together with PCR fragment bvgASup/F1, into XbaI- and EcoRI-digested pUC18 vector DNA. The resulting plasmids, pUC-bvgASup \textit{G7702} and pUC-bvgASup \textit{ATCC 51541} were digested with Xbal and EcoRI and the resulting 4-7 kbp DNA fragments were finally inserted into plasmid pRK415, giving rise to plasmids pRK-bvgASup \textit{G7702} and pRK-bvgASup \textit{ATCC 51541}, respectively.

\section*{Construction of a bvgASup mutant of \textit{B. holmesii G7702.}}

For the construction of the suicide plasmid pSS1129-bvgASup-kan\textsuperscript{3}, a 1-8 kbp DNA fragment comprising the bvgASup gene and part of its 5' and 3' flanking regions was PCR amplified from genomic DNA of \textit{B. holmesii G7702} using primer pair bvgASup-kan\textsuperscript{3}/bvgASup-kan\textsuperscript{3}, which provided BamHI restriction sites at the 5' and 3' termini. This PCR fragment was cloned into pACYC184, and the resulting plasmid was digested with PstI, thereby deleting 354 bp from bvgASup because of the presence of three PstI restriction sites (at positions 206, 467 and 560 with respect to the bvgASup start codon) in the bvgASup gene. Subsequently, a kanamycin-resistance cassette, excised by PstI cleavage from pUC4K, was inserted into the PstI-digested plasmid pACYC-bvgASup, yielding plasmid pACYC-bvgASup-kan\textsuperscript{3}. The bvgASup-kan\textsuperscript{3} fragment was excised with BamHI and ligated into the suicide vector pSS1129, giving rise to plasmid pSS1129-bvgASup-kan\textsuperscript{3}, which was subsequently transformed into \textit{E. coli} SM10. pSS1129-bvgASup-kan\textsuperscript{3} was then conjugated into \textit{B. holmesii G7702} using \textit{E. coli} SM10 as donor strain. Allelic exchange resulted in the \textit{B. holmesii} strain G7702/bvgA::kan. The correct insertion of the kanamycin-resistance cassette into the bvgASup gene of the kanamycin-resistant exconjugants was proven by PCR reactions with primers flanking the integration site and by Southern blot analysis.

\section*{Construction of \textit{B. holmesii} and \textit{B. pertussis} strains containing a plasmid with a fusion of the upstream region of bvgASup to the gfp reporter gene.}

A DNA fragment containing the promoterless gfp-mut2 gene (Cormack \textit{et al.}, 1996) was excised with Xbal and PstI from plasmid pKEN and ligated into plasmid pUC18, giving rise to plasmid pUC-gfp. A 455 bp DNA fragment containing the upstream region of the bvgASup locus was PCR amplified from genomic DNA of \textit{B. holmesii G7702} using primer pair bvgASup-up5/bvgASup-up3, thereby introducing BamHI and Xbal restriction sites at the 5' and 3' termini, respectively, and the fragment was cloned into pUC-gfp, resulting in plasmid pUC-bvgASup-up-gfp. The bvgASup-up-gfp fragment was then excised by BamHI- and PstI-digestion and was subsequently ligated into plasmid pMMB208. In the resulting plasmid pMMB-bvgASup-up-gfp, the bvgASup-up-gfp promoter fusion is located in the opposite orientation to the plasmid-borne tac promoter. pMMB-bvgASup-up-gfp was subsequently transformed into \textit{E. coli} SM10 and transferred by conjugation into various \textit{B. holmesii} and \textit{B. pertussis} strains.

\section*{Construction of plasmids expressing recombinant BvgA\textsubscript{BP}, BvgS\textsubscript{BP}, and BvgA\textsubscript{BP} proteins, and protein purification.}

The construction of pQE-BvgSTRO and pCyt-BvgSTRO has been described by Perraud \textit{et al.} (1998). For the generation of plasmid pQE-BvgASup, a DNA fragment including the complete bvgASup gene (621 bp) was amplified from genomic DNA of \textit{B. holmesii G7702} using primer pair bvgASup1-BamHI/bvgASup-KpnI, thus introducing BamHI and KpnI sites at its 5' and 3' ends respectively. This PCR fragment was ligated into BamHI/KpnI-digested pQE30 vector DNA, creating a N-terminal His\textsubscript{tag}. Recombinant His\textsubscript{tag}-BvgASup and His\textsubscript{tag}-BvgSTRO, encoded on plasmids pQE-BvgASup and pQE-BvgSTRO, respectively, were expressed in \textit{E. coli} M15 cells and the proteins were purified by affinity chromatography on Ni\textsuperscript{2+}-nitrilotriacetic acid agarose (Qiagen) essentially as described by Perraud \textit{et al.} (1998). Native BvgASup encoded on plasmid pCyt-BvgASup was over-expressed in \textit{E. coli} DH5\textalpha cells and the protein was purified by affinity chromatography on heparin-Sepharose CL-6B (Pharmacia) as described by Perraud \textit{et al.} (1998). In vitro-phosphorylation assays with the recombinant proteins were performed as described by Bock & Gross (2002).

\section*{ Primer extension experiments.}

Total RNA was prepared from bacteria grown in liquid culture as described previously (Gross & Rappuoli, 1989). Primer extension experiments were carried out essentially as described by Scarlato \textit{et al.} (1991) with primer oligonucleotides fhaB-PE, bvgA\textsubscript{BP}-PE and gfp-PE (Table 2). Sequencing reaction mixtures, with plasmids pSL-bvgASup \textit{G7702} and pUC-bvgASup-up-gfp as template DNA and the appropriate oligonucleotide primer, were analysed on 6% urea-polyacrylamide gels and used as standards for determination of the transcription initiation sites.

\section*{ Gel retardation experiments.}

A 77 bp DNA fragment encompassing the BvgA\textsubscript{BP}-P binding site of the fhaB promoter was amplified by PCR using primer pair fhaBup5/fhaBup3 and plasmid pProm67 as template. The PCR fragment was 5'-end labelled with [\textsuperscript{32}P]ATP using T4 polynucleotide kinase (MBI) and purified using the QIAquick Nucleotide Removal Kit (Qiagen). The recombinant BvgA proteins were diluted in 1× dilution buffer (2 mM MgCl\textsubscript{2}, 50 mM KCl, 0.1% Igepal CA 630, 10 mM DTT) and were phosphorylated by incubation with 50 mM acetyl phosphate (Sigma) for 20 min at room temperature. Increasing amounts of the proteins were added to approximately 15 000 c.p.m. of the labelled DNA probe in 20 μl of 1× binding buffer (10 mM Tris/HCl, pH 8, 10 mM KCl, 5 mM EDTA, 1 mM DTT, 10% glycerol, v/v). The samples were incubated for 20 min at room temperature and were then loaded onto a non-denaturing 4% polyacrylamide gel (Sambrook & Russell, 2000). Gels were run for 2-5 h at 150 V and subsequently the dried gels were autoradiographed.

\section*{DNase I footprinting.}

DNase I footprinting experiments were performed essentially as described by Dickneite \textit{et al.} (1998). A 253 bp DNA fragment containing part of the upstream region of the bvgASup locus was PCR amplified from chromosomal DNA of \textit{B. holmesii G7702} using primer pair FPI-3/FPI-5. The purified promoter fragment was cloned into plasmid pGEM-T. The resulting plasmid pGEM-T-FP was digested with BamHI and 5'-end labelled with [\textsuperscript{32}P]ATP using T4 polynucleotide kinase. The labelled promoter fragment was excised from the plasmid by KpnI digestion, purified by gel electrophoresis and eluted in 3 ml elution buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA, 300 mM sodium acetate, 0.2% SDS). The eluted probe was then extracted with phenol/chloroform (1:1, v/v) and ethanol precipitated. Binding reaction mixtures contained various concentrations of protein and approximately 100 000 c.p.m. of labelled DNA probe in 50 μl of 1× binding buffer (10 mM Tris/HCl, pH 8, 2 mM MgCl\textsubscript{2}, 0.1 mM CaCl\textsubscript{2}, 1 mM DTT, 10% glycerol, v/v). The samples were incubated for 20 min at room temperature and then the nucleolytic reactions were initiated by the addition of UDNase I in 1× binding buffer. After 1 min, digestions were terminated by the addition of 140 μl stop buffer (192 mM sodium acetate, 0.14% SDS, 62 μg ml\textsuperscript{-1} yeast tRNA). The samples were extracted with phenol/chloroform.
(1:1, v/v), ethanol precipitated and run on a 6% polyacrylamide-urea sequencing gel. A G+C sequence reaction was also conducted in parallel with the labelled DNA probe (Maxam & Gilbert, 1977) and electrophoresed on the same gel.

RESULTS

Characterization of the bvgAS loci of the ‘new’ Bordetella species

To clone the bvgA genes of B. holmesii (BH), B. hinzii (BHZ) and B. trematum (BT), a PCR approach, with degenerate primers deduced from highly conserved regions of the receiver domain and from the output domain of BvgA from B. pertussis (BP), was used. The obtained DNA fragments of 285 or 237 bp were used as probes to screen partial genomic libraries of the respective organisms or were extended by genome walking. Assembly of the resulting DNA sequences yielded the entire bvgAS locus, and 873 bp and 1373 bp from its upstream and downstream regions in the case of B. holmesii. In the case of B. trematum, 1068 bp from the upstream region of the bvgAS locus, and 3142 bp encoding BvgA and the N-terminal 839 amino acids of BvgS were obtained. From the B. hinzii bvgAS locus, a partial sequence, comprising a fragment encoding the N-terminal 166 amino acids of BvgA and 660 bp from the upstream region, was determined.

The genome organization of the bvgAS loci of the ‘new’ Bordetella species differs from that of the members of the B. bronchiseptica cluster. In B. holmesii, B. hinzii and B. trematum, a gene (orfX) encoding a putative response regulator was detected at a distance of about 400 bp upstream from bvgA in the same transcriptional direction, while in the members of the B. bronchiseptica cluster the fhaB gene encoding filamentous haemagglutinin (FhaB) is located upstream of bvgA (Parkhill et al., 2003). Also, in B. avium, an ORF encoding a homologous response regulator was found upstream of bvgA; this ORF was annotated by Spears et al. (2003) as an orthologue of the vieA gene of V. cholerae. Furthermore, no orthologue of the bvgR gene, which is located downstream of bvgAS in the genomes of the members of the B. bronchiseptica cluster is present within the analysed region downstream of bvgAS in B. holmesii. The lack of a bvgR orthologue in the downstream region has already been described for B. avium (Spears et al., 2003), therefore suggesting that the genome organization of the bvgAS locus is conserved in B. avium and the ‘new’ Bordetella species (Fig. 1).

As was reported for B. avium (Spears et al., 2003), the nucleotide sequences of the bvgAS genes of the ‘new’ Bordetella species exhibit only a low degree of similarity with the orthologous genes of the members of the B. bronchiseptica cluster, with the pairwise identity between the bvgA and bvgS sequences of B. holmesii and the orthologues from B. pertussis being 72.7% and 59.9%, respectively. However, the amino acid sequences of the BvgA proteins of B. holmesii or B. trematum and BvgA from B. pertussis are highly similar in the receiver and output domains (74.8% and 86.1% mean pairwise identity, respectively), while the linker region connecting the two functional domains is less conserved (34.5% pairwise identity). Similarly, the alignment of the BvgS orthologues of B. holmesii and B. pertussis showed a high similarity in the cytoplasmic transmitter, receiver and HPT domains (52.9% identity), but much less sequence conservation in the periplasmic domain, which is believed to be involved in signal perception (39.5% identity).

Interestingly, DNA sequence analysis of the bvgA gene of the B. holmesii type strain ATCC 51541 (bvgA_H ATCC 51541) revealed the insertion of an adenosine residue at nucleotide position 212 which leads to a frameshift mutation that causes the premature termination of the encoded protein. Therefore, we sequenced the bvgA_H gene of three additional B. holmesii strains which were isolated independently from each other in different geographical regions and at different times. In one of these strains, B. holmesii No1, we found the identical mutation to that seen in the type strain, while the bvgA_H genes of the other two isolates, B. holmesii G7702 and G8341, are intact. This observation is reminiscent of the phenomenon of phase variation in B. pertussis and B. bronchiseptica and, therefore subsequently in this paper B. holmesii isolates with an intact bvgA_H

Fig. 1. Schematic representation of the genomic organization of the bvgAS loci of B. pertussis, B. avium, B. holmesii, B. trematum and B. hinzii. Arrows represent coding sequences; non-coding DNA is indicated as thin lines. Rectangles indicate incomplete sequencing of the bvgS and bvgA genes of B. trematum and B. hinzii, respectively. The sequence of orfX was not determined completely. The complete genome sequence of B. pertussis and B. avium was determined by the Sanger Centre (Parkhill et al., 2003; http://www.sanger.ac.uk/Projects/B_avium). The figure is not drawn to scale.
The cytoplasmic signalling domains of BvgS Bh and BvgA Bh are functionally interchangeable

It has been shown already that the bvgAS loci of B. pertussis and B. bronchiseptica can replace each other in vivo (Monack et al., 1989; Martínez de Tejada et al., 1996). To investigate whether the bvgAS Bh genes of B. holmesii are able to functionally complement B. pertussis bvgAS mutants, we introduced plasmid pRK-bvgAS Bh G7702, containing the orfX-bvgAS intergenic region and the entire bvgAS locus of B. holmesii G7702 (bvgAS Bh G7702) into the B. pertussis strains 359 and 347 by conjugation. BP 359 contains a polar Tn5 insertion in the bvgA Bh gene and is therefore unable to express both the BvgA Bh and the BvgA Sp proteins. In BP 347, the bvgA Bh gene is disrupted, resulting in the low-level expression of BvgA Bh due to the presence of a weak constitutive promoter upstream of bvgA Sp (Scarlatò et al., 1991); however, BvgA Sp is present in its non-phosphorylated and therefore inactive state (Fig. 2a).

Expression of the B. holmesii bvgAS Bh locus in BP 359 (pRK-bvgAS Bh G7702) was proven by RT-PCR experiments and Western blotting with a BvgA Sp-specific polyclonal antibody which also recognizes BvgA Sp (data not shown). BP 359 (pRK-bvgAS Bh G7702) and BP 347 (pRK-bvgAS Bh G7702) were compared with the positive control strain BP 347 (pLA57vir) and BP 359 (pLA57vir), which are complemented with the wild-type bvgAS Bh locus for the ability to express the virulence determinants FhaB and Cya (adenylate cyclase toxin). Strain BP 347 (pRK-bvgAS Bh G7702) but not BP 359 (pRK-bvgAS Bh G7702) formed small haemolytic colonies on BG-blood agar plates and produced FhaB, as determined by immunoblot analysis (Fig. 2b). The failure of strain BP 359 (pRK-bvgAS Bh G7702) to activate fhaB transcription was also confirmed by primer extension experiments with an fhaB-specific oligonucleotide primer and RNA prepared from BP 359 (pRK-bvgAS Bh G7702) (data not shown). These data suggest that, despite the high degree of sequence similarity, BvgA Bh can not functionally substitute for BvgA Sp in B. pertussis in vivo, while BvgA Sp can replace BvgA Bh in the phosphorylation of BvgA Sp.

To confirm that the successful complementation in BP 347 (pRK-bvgAS Bh G7702) was due to cross-phosphorylation between BvgA Sp and BvgA Bh, plasmid pRK-bvgAS Bh ATCC 51541, carrying the bvgAS Bh locus of the bvgA phase variant B. holmesii ATCC 51541, was introduced into BP 347 by conjugation. As expected, the resulting strain BP 347 (pRK-bvgAS Bh ATCC 51541) was haemolytic on BG blood agar plates and expressed FhaB (data not shown). In keeping with these results, efficient cross-phosphorylation between the purified BvgA Sp and BvgA Bh proteins was also observed in vitro (data not shown).

Since it is well known that high concentrations of MgSO4 inhibit the histidine kinase activity of BvgA Sp (Melton & Weiss, 1989), strain BP 347 (pRK-bvgAS Bh ATCC 51541) was analysed for the activity of histidine kinase BvgA Sp in the presence of MgSO4. The effect of MgSO4 on the phosphorylation status of BvgA Bh ATCC 51541, and consequently on the expression of the virulence genes, was investigated by primer extension experiments using the fhaB-specific primer and RNA extracted from BP 347 (pRK-bvgA Bh ATCC 51541) cells cultured in the presence or absence of 50 mM MgSO4. As shown in Fig. 2(c), addition of MgSO4 to the culture medium completely repressed fhaB transcription in the positive control strain BP 347 (pLA57vir), while transcription of fhaB was only slightly reduced under these growth conditions in BP 347 (pRK-bvgA Bh ATCC 51541). Taken together these data demonstrate that the cytoplasmic signalling domains of BvgA Sp and BvgA Bh are functionally interchangeable, while differences exist regarding the function of their N-terminal signal-perception domains.

BvgA Bh binds in vitro to the orfX-bvgA Bh intergenic region but not to the BvgA Sp binding site of the fhaB promoter of B. pertussis

The results of the complementation experiments suggest that the failure of plasmid pRK415-bvgA Bh G7702 to restore virulence gene expression in the B. pertussis mutant BP 359 is due to the inability of BvgA Bh to bind to the fhaB and cya promoters. Therefore, we investigated, by gel retardation experiments, the in vitro binding of BvgA Bh to a 77 bp DNA fragment containing the well-characterized BvgA Sp binding site of the promoter region of the B. pertussis fhaB gene. A clear band shift of the fhaB probe was observed with the purified BvgA of B. pertussis, irrespective of whether the protein was in vitro phosphorylated with acetyl phosphate prior to the binding assay, as has been described by Zu et al. (1996) (Fig. 3b). In contrast, no binding to the fhaB promoter fragment could be detected with either the unphosphorylated or the phosphorylated BvgA Bh protein (Fig. 3a), suggesting that BvgA of B. holmesii is unable to recognize canonical BvgA Sp-dependent promoters. In keeping with this hypothesis, we could not detect binding of BvgA Bh to the BvgA Sp promoter region in gel retardation experiments (data not shown).

As transcription of the bvgAS locus is autoregulated in the members of the B. bronchiseptica cluster (Scarlatò et al., 1990; Karimova & Ullmann, 1997), we analysed whether BvgA Bh is able to bind the intergenic region between orfX and bvgA Bh in B. holmesii. DNease I footprint experiments, with increasing amounts of BvgA Bh, were performed on a cloned 253 bp DNA segment spanning from nucleotide position +20 to −232 with respect to the translational start site of bvgA Bh. Addition of 180 ng in vitro-phosphorylated
BvgABH (BvgABH-P) resulted in a region of DNase I protection ranging from position +270 to +2195 with respect to the start codon of \textit{bvgA}_{BH}, with the appearance of a regular pattern of hypersensitive sites every 10 to 11 nucleotides (Fig. 4). No further extension of the protected region was observed upon the addition of higher amounts of BvgABH-P. This was tested by using additional overlapping DNA probes covering an entire region ranging from position +81 to +2313 with respect to the \textit{bvgA}_{BH} start codon (data not shown). Binding of unphosphorylated BvgA\textit{BP} to the \textit{bvgA}_{BH} promoter probe could not be detected (Fig. 4). Interestingly, a virtually identical binding pattern was observed when in \textit{vitro}-phosphorylated BvgA from \textit{B. pertussis} was added to the \textit{bvgA}_{BH} promoter probe (data not shown). As in the case of BvgA\textit{BP}, unphosphorylated BvgA\textit{BP} was unable to bind to the \textit{bvgA}_{BH} promoter region (data not shown).

Careful \textit{in silico} analysis of the 125 bp region which is protected from DNase I digestion in the \textit{orfX-bvgA}_{BH} intergenic region revealed the presence of four 14 bp sequence motifs (IR1–IR4) which are arranged at a regular spacing of 20–21 bp from each other. These sequence motifs bear a certain resemblance to the consensus heptanucleotide inverted-repeat sequence which constitutes the primary binding site for BvgA\textit{BP} in BvgA\textit{BP}-dependent \textit{B. pertussis}.
pertussis promoters, as one half site of each sequence motif matches the BvgA BP consensus half site motif in six (IR1, IR2), or four (IR3, IR4) out of seven positions (Fig. 5). The second half site, however, does not show significant similarity to the BvgA BP consensus binding site. Therefore, the 14 bp sequence motifs, which all exhibit similar binding affinities for BvgABH at least under the conditions of in vitro DNA binding, might represent the primary binding sites of BvgABH in the orfX-bvgASBH intergenic region.

Taken together, the results on the binding of BvgA BH to the orfX-bvgASBH intergenic region suggest an autoregulatory role for BvgA BH in the expression of the BvgAS two-component system of B. holmesii.

**BvgA BH activates transcription of the bvgASBH locus in B. holmesii**

To analyse whether the bvgAS locus of B. holmesii is autoregulated, primer extension experiments were performed with RNA extracted from B. holmesii strain G7702 and the isogenic bvgA knockout mutant G7702/bvgA BH :: kan, using a bvgA BH-specific oligonucleotide. As shown in Fig. 6(a), two transcriptional start sites could be identified at positions −20 (S1) and −34 (S2) with respect to the translational start codon of bvgA BH. Upstream of both start sites, putative −10 regions are present which exhibit one (P2: CATAAT) and two (P1: TAAAAC) mismatches compared to the −10 consensus promoter element of E. coli. These putative −10 elements are located at a distance of 27 and 42 bp, respectively, from the IR1 sequence motif, within the region which is protected from DNase I digestion.

**Fig. 3.** Binding of BvgA BH and BvgA BP to the BvgA BP binding site of the B. pertussis fhaB promoter. (a) A radiolabelled 77 bp PCR fragment containing the BvgA BP binding site of the fhaB promoter of B. pertussis was incubated with 80, 160, 240, 360 and 600 ng of purified BvgA BH which was phosphorylated in vitro (lanes 2–6) and with unphosphorylated BvgA BH (lanes 7–11), respectively. (b) The same radiolabelled fhaB promoter fragment was incubated with 80, 160, 240, 360 and 600 ng of in vitro phosphorylated (lanes 2–6) and unphosphorylated (lanes 7–11) BvgA BH, respectively. Lane 1 on both panels contains the radiolabelled DNA probe. The reaction mixtures were run on a non-denaturing 4 % polyacrylamide gel. F, free DNA; C, DNA–protein complex.

**Fig. 4.** Binding of BvgA BH to the intergenic region between orfX and bvgASBH. DNase I footprint experiments were performed on a 253 bp BamHI–KpnI DNA fragment from plasmid pGEM-T-FP labelled at its BamHI site. The 5′-labelled probe was incubated with 180, 360 and 600 ng in vitro-phosphorylated BvgA BH (BvgA BH-P; lanes 3–5, respectively) or the same amounts of unphosphorylated BvgA BH (lanes 6–8, respectively). No protein was added to the reaction mixture loaded in lane 2. Lane 1 is a G+A sequencing reaction on the DNA probe used as a size marker (Maxam & Gilbert, 1977). Numbers on the left indicate the distance from the translational start codon of bvgA BH. The vertical bar on the right indicates the area of DNase I protection.
The regulatory BvgAS two-component system is the master regulator of virulence gene expression in the members of *B. pertussis*. The binding of the RNA polymerase to this specific promoter located in the *bvgAS* locus of *B. holmesii*, which is different from the promoters directing transcription of the *bvgAS* gene in *B. pertussis* and *B. holmesii* upstream region, interferes with the binding of the RNA polymerase to this *B. pertussis*-specific promoter located in the *bvgASBH* upstream region. Interestingly, a transcriptional start site differing only slightly (position −130) from the one used in *B. pertussis* is observed when transcription of the *bvgASBH*-specific promoter fusion is analysed in *B. pertussis* (data not shown).

**DISCUSSION**

The regulatory BvgAS two-component system is the master regulator of virulence gene expression in the members of *B. pertussis*. The...
the B. bronchiseptica cluster which cause respiratory tract infections in man and other mammals, and has been demonstrated to play a critical role in the establishment and maintenance of disease (Cotter & DiRita, 2000; Bock et al., 2001). Since the ‘new’ members of the genus Bordetella, in particular B. holmesii, are increasingly recognized to be associated with human diseases, we set out to clone putative orthologues of the bvgAS genes of these bacteria as a first approach to characterize their pathogenic potential.

By PCR with degenerate oligonucleotide primers derived from the BvgA sequence of B. pertussis, we obtained bvgA-specific DNA fragments from the three organisms; these fragments were used as probes for the cloning of the complete bvgAS locus from B. holmesii and of partial sequences of bvgAS from B. trematum and B. hinzii. At the DNA level, the bvgAS genes of the three ‘new’ Bordetella species show only limited sequence similarity with their orthologues from the members of the B. bronchiseptica cluster, explaining our previous failure to prove their presence by DNA hybridization experiments with B. pertussis-specific DNA-probes (Gerlach et al., 2001). However, as far as they were determined, the deduced amino acid sequences of BvgAS were found to be conserved between all species including the bird pathogen B. avium whose bvgAS genes have recently been cloned (Spears et al., 2003). As expected, sequence similarity is highest in the two-component signalling modules but is less pronounced in the recently characterized linker region of BvgA (Bock et al., 2001) and the sensory domain of BvgS. Clearly the BvgAS proteins of the ‘new’ Bordetella species and of B. avium are more closely related to each other than to the orthologous proteins of the members of the B. bronchiseptica cluster (Table 3).

Similarly, the genomic organization of the bvgAS loci of the ‘new’ Bordetella species and of B. avium seems to be identical, but differs from the members of the B. bronchiseptica cluster. In the ‘new’ Bordetella species and in B. avium, a gene (orfX) encoding a putative response regulator protein was observed upstream of bvgAS, and downstream no orthologue of the gene encoding the BvgR protein, which is involved in regulating the virulence repressed genes (vrg) in the members of the B. bronchiseptica cluster, is present in B. holmesii and B. avium (Fig. 1). The upstream regulator is also present in B. bronchiseptica and B. parapertussis, although at a different location on the chromosome, while it is missing in B. pertussis.

Fig. 6. Determination of the transcriptional start site of bvgASBH by primer extension analysis. Equal amounts of total RNA extracted from B. holmesii G7702 (lane 1) and B. holmesii G7702/ bvgASBH::kan (lane 2) were hybridized with radiolabelled oligonucleotide bvgA BH-PE. Transcriptional start points are indicated by arrows. A part of the promoter sequence is shown on the left. The corresponding putative −10 promoter elements are boxed. The sequencing reaction (lanes A, C, G and T) was performed using oligonucleotide bvgA BH-PE and plasmid pSL-bvgASBH G7702 as template. (b) Immunoblot analysis of equal amounts of proteins prepared from E. coli(pUC-bvgASBH-up-gfp) (lane 1), B. holmesii G7702 (lane 2), B. holmesii G7702(pMMB-bvgASBH-up-gfp) (lane 3) and B. holmesii G7702/bvgASBH::kan(pMMB-bvgASBH-up-gfp) (lane 4). The membrane was probed with a polyclonal antiserum directed against GFP. The arrow indicates the positions of the GFP-specific band.

Fig. 7. Expression of the gfp reporter gene fused to the bvgASBH promoter in B. pertussis. (a) Immunoblot analysis of GFP expression in E. coli(pUC-bvgASBH-up-gfp) (lane 1), BP Ti (lane 2), BP Tk(pMMB-bvgASBH-up-gfp) (lane 3), BP 347 (lane 4), BP 347(pMMB-bvgASBH-up-gfp) (lane 5), BP 359 (lane 6), BP 359(pMMB-bvgASBH-up-gfp) (lane 7). Equal amounts of protein were blotted and were probed with a polyclonal antiserum directed against GFP. The arrow indicates the positions of the GFP-specific band. (b) Determination of the transcriptional start site of the gfp reporter gene fused to the upstream region of bvgASBH of B. holmesii by primer extension analysis. Equal amounts of total RNA extracted from B. pertussis 347(pMMB-bvgASBH-up-gfp) (lane 1) and B. pertussis 359(pMMB-bvgASBH-up-gfp) (lane 2) were hybridized with radiolabelled oligonucleotide gfp-PE. The transcriptional start point is indicated by an arrow. A part of the bvgASBH promoter sequence is shown on the left. The corresponding putative −10 promoter element is boxed. The sequencing reaction (lanes A, C, G and T) was performed using oligonucleotide gfp-PE and plasmid pUC-bvgASBH-up-gfp as template.
Unexpectedly, 16S rDNA sequence analysis had suggested that the closest phylogenetic relationship was between B. pertussis and B. holmesii (Weyant et al., 1995; Gerlach et al., 2001); however, in agreement with comparisons of the amino acid sequences of conserved proteins (von Wintzingerode et al., 2002), our characterization of the bvgAS loci clearly classifies B. holmesii as proximate to the species B. avium, B. hinzii and B. trematatum. Interestingly, the bvgA sequences of two out of four independent clinical isolates of B. holmesii contained an identical point mutation causing a frameshift that results in the premature termination of the encoded protein. This is reminiscent of phase variation in B. pertussis and B. bronchiseptica leading to the loss of virulence properties upon in vitro passaging of the respective strains due to mutations in the bvgS genes (Monack et al., 1989; Stibitz et al., 1989). However, phase variants of these organisms have apparently never been isolated from infected hosts. Since inactivation of the bvgA gene in B. holmesii G7702 had no obvious effect on growth and colony morphology of the mutant, the possibility that frameshift mutations in strains ATCC 51541 and No1 occurred after their isolation from the infected patients can not be ruled out. But, since the site of the mutation is not associated with a homo- or dinucleotide tract which could cause slipped-strand mispairing, it seems rather unlikely that the identical insertion mutation should have occurred in both strains as a consequence of in vitro culture. The apparent isolation of bvgA mutants from infected individuals would clearly argue against a role of the BvgAS_BH two-component system in the regulation of important virulence traits in B. holmesii, while inactivation of bvgAS_BA caused a clear attenuation of the virulence of B. avium in turkey pouls (Spears et al., 2003). Interestingly, Njamkepo et al. (2000) reported differences in the protein hybridization patterns of different B. holmesii isolates, including the type strain, ATCC 51541, and G7702, when the bacterial lysates were probed with sera from infected individuals, and therefore they suggested that the phenomenon of phase variation might exist in B. holmesii. However, the observed differences in the protein patterns do not correlate with the presence or absence of a functional BvgA_BH protein as predicted by our sequence analysis of the same strains.

The observation that the bvgAS_BH locus is able to restore virulence gene expression in the B. pertussis bvgS mutant BP 347 but not in the bvgAS mutant BP 359 suggested cross-phosphorylation between the BvgS_BH histidine kinase and BvgA_BP (Fig. 2). Similarly, cross-phosphorylation was also demonstrated to occur between BvgS_BH and BvgA_BH in vitro (data not shown). Therefore, the cytoplasmic signalling domains of BvgS_BH and BvgS_BP, showing 52-9 % identity, are functionally interchangeable in the recognition of the orthologous BvgA response regulator proteins, whose receiver domains are 76.2 % identical. In the genetic background of BP 347, BvgS_BH is only slightly responsive to the presence of 50 mM MgSO₄, which causes phenotypic modulation in the wild-type B. pertussis strain TI. Therefore, in accordance with the observation that the periplasmic input domains of BvgS_BH and BvgS_BP show a far lower degree of similarity (39.5 % identity) than the cytoplasmic domains, signal perception of the BvgS proteins from B. holmesii and B. pertussis seems to be different. Similarly, in the genetic background of B. bronchiseptica, the function of the BvgS_BP protein was indistinguishable from BvgS_BB when the expression of virulence factors, under either standard in vitro culture conditions or in vivo growth in an animal model, was investigated, but the sensitivity to modulating agents in vitro was different between BvgS_BB and BvgS_BP (Martinez de Tejada et al., 1996). BvgS_BB and BvgS_BP are highly similar (87 % overall identity), but, as seen with BvgS_BH and BvgS_BP, most of the non-conservative amino

### Table 3. Similarity between the BvgA and BvgS proteins from different Bordetella species

Pairwise identity and similarity (in parentheses) between the two-component proteins from the respective Bordetella species are given as percentages. In the case of BvgS, pairwise identity and similarity between the cytoplasmic signalling domains of the respective sensor proteins is also shown.

<table>
<thead>
<tr>
<th></th>
<th>B. pertussis</th>
<th>B. avium</th>
<th>B. holmesii</th>
<th>B. trematatum</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Similarity in BvgA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. pertussis</td>
<td>–</td>
<td>75.5 (82-8)</td>
<td>76.0 (84-8)</td>
<td>75.0 (83-2)</td>
</tr>
<tr>
<td>B. avium</td>
<td>–</td>
<td>–</td>
<td>93.2 (94-2)</td>
<td>91.8 (94-2)</td>
</tr>
<tr>
<td>B. holmesii</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>92.3 (95-7)</td>
</tr>
<tr>
<td>B. trematatum</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>B. pertussis</th>
<th>B. avium</th>
<th>B. holmesii</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Similarity in BvgS/BvgS_cyt</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. pertussis</td>
<td>–</td>
<td>45.0 (54-0)/51.7 (61-0)</td>
<td>46.8 (54-9)/52.9 (61-3)</td>
</tr>
<tr>
<td>B. avium</td>
<td>–</td>
<td>–</td>
<td>63.0 (70-2)/66.4 (73-1)</td>
</tr>
<tr>
<td>B. holmesii</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

**Data provided in parentheses.**
acid substitutions occur in the periplasmic domain which has been shown to be solely responsible for the observed differences in the sensitivity to modulating signals (Martinez de Tejada et al., 1996).

In contrast to the cytoplasmic signalling domains of BvgSBP and BvgS BH, the BvgA response regulators are not functionally interchangeable between B. pertussis and B. holmesii. The observations that the B. pertussis bvgAS mutant BP 359 could not be complemented to virulence gene expression from the bvgAS locus of B. holmesii in trans (Fig. 2) and that BvgA BH did not bind to the BvgA BP binding sites of the B. pertussis fhaB (Fig. 3) and bvgA (data not shown) promoters in vitro, indicate that BvgA BH is unable to productively interact with BvgA-dependent promoters of B. pertussis which are characterized by the presence of multiple BvgA BP binding sites. The fhaB promoter contains an imperfect heptanucleotide inverted-repeat sequence with abutting half-sites centred at position −108.5 relative to the transcriptional start site, which is the high-affinity binding site of a BvgA BP-P dimer, as well as two additional low-affinity binding sites centred at position −67.5 and directly adjacent to the −35 region (Boucher et al., 2001, 2003). These secondary binding sites show a very limited degree of similarity to the high-affinity heptanucleotide inverted-repeat motif, but binding of BvgA BP-P to the low-affinity binding sites is required for full transcriptional activation of the fhaB promoter (Boucher et al., 1997, 2001).

Similarly, the ptx promoter harbours two inverted heptad repeats in the region ranging from position −167 to −123 relative to the transcriptional start site. These repeats match the fhaB high-affinity binding motif in five of seven positions, and cooperative binding of multiple BvgA BP-P dimers to the DNA downstream of these primary binding sites is necessary for the activation of ptx transcription (Boucher & Stibitz, 1995; Marques & Carbonetti, 1997). Differences in the affinity of the BvgA BP binding sites in the fhaB and ptx promoters, resulting in the requirement of higher concentrations of BvgA BP-P for transcriptional activation of ptx, account for the different temporal pattern of expression of these virulence genes (Scarlato & Rappuoli, 1991). Here we demonstrate that in B. holmesii transcription of bvgAS BH is under control of two overlapping promoters which are positively regulated by BvgA BH, since transcription of bvgAS BH and expression of a gfp reporter gene which was fused to the bvgAS BH promoter region were reduced in the bvgAS BH mutant G7702/bvgAS BH::kan (Fig. 6). However, basal BvgA BH-independent transcription from these promoters was also detected in G7702/bvgAS BH::kan, and this might be responsible for low-level expression of BvgAS BH under conditions in which the BvgA BH histidine kinase is inactive. The moderate difference in the amount of bvgAS BH transcript in strains G7702 and G7702/bvgA BH::kan might indicate that, in contrast to BvgS BP, BvgS BH is not fully activated under the standard in vitro culture conditions tested so far.

Interestingly, the region upstream of the regulated bvgAS BH promoters, which is protected in DNase I footprint experiments with in vitro-phosphorylated BvgA BH, contains four 14 bp sequence motifs (IR1–IR4) in a regular spacing of 20–21 bp, which show similarity to the heptanucleotide inverted-repeat sequence that has been deduced as consensus motif for the primary binding site for BvgA BP in BvgA BP-dependent B. pertussis promoters (Fig. 5). This arrangement of binding sites might suggest end-to-end binding of BvgA BH dimers to the same face of the DNA helix. The sequence motifs IR1 and IR2, showing the highest degree of similarity to the BvgA BP consensus half-site motif T/A-T-T-C-C/T-T-A, are located proximal to the transcriptional start site of bvgAS BH, while IR3 and IR4, which match the sequence of the consensus binding site in four of seven positions, are located further upstream. This architecture of putative high- and low-affinity BvgA BP binding sites differs from the fhaB and ptx promoters, but resembles the B. pertussis core bvgR promoter, which, for activity, requires binding of BvgA BP-P to a secondary binding site located at a distance of 22 bp upstream from the primary binding site, which is centred at position −53.5 proximal to the transcriptional start site. Interestingly, the BvgA BP binding motifs in the bvgR promoter also consist of one half-site with high-scoring similarity to the BvgA BP consensus binding motif in combination with a low-scoring half-site (Merkel et al., 2003). However, DNase I footprint experiments did not provide indications for differences in the binding affinities of sequence motifs IR1–IR4 in the bvgAS BH promoter since a sharp onset of protection, spanning the complete region from position −70 to −195 relative to the bvgAS BH start codon, was observed upon addition of low amounts of BvgA BH. Considering the striking similarity of the bvgAS BH promoter region to BvgA-dependent promoters of B. pertussis and the fact that in vitro binding of BvgA BP-P to this promoter is virtually indistinguishable from BvgA BH, the inability of BvgA BH to interact with B. pertussis promoters remains unexplained.

In the B. pertussis bvgAS mutant BP 359 containing the bvgAS BHup-gfp promoter fusion, transcription of the gfp reporter gene is directed from a promoter different to the overlapping P1 and P2 promoters in B. holmesii. Transcription from this promoter starts within the region of BvgA BH and BvgA BP-binding, unless the detected transcript is the defined processing product of a longer mRNA transcribed from a cryptic promoter located further upstream or in the plasmid backbone (Fig. 5, Fig. 7). In the B. pertussis wild-type strain TI, BvgA BP does not stimulate transcription from the overlapping bvgAS BH-dependent P1 and P2 promoters, but instead binding of BvgA BP-P to the upstream region of bvgAS BH seems to interfere with transcription from the alternative promoter since no expression of the gfp reporter gene could be detected in Tl(pMMB-bvgA BHup-gfp). Although in vitro binding of unphosphorylated BvgA BP to the bvgAS BH promoter region was not observed, expression of gfp was also strongly reduced in the bvgS mutant BP 347(pMMB-bvgAS BHup-gfp) as compared to BP 359(pMMB-bvgAS BHup-gfp) (Fig. 7).
should be noted that despite the inhibitory effect of BvgA BP on transcription from the bvgASBH upstream region in B. pertussis, low-level expression of BvgSBRH was sufficient to restore virulence gene expression in BP 347(pRK-BvgASBRH G7702). We are currently trying to identify the members of the BvgA regulon of B. holsøesi. The characterization of additional BvgABRH-dependent promoters will help to unravel the molecular basis of the striking differences in promoter recognition observed with the highly homologous BvgA proteins of B. holsøesi and B. pertussis.

**ACKNOWLEDGEMENTS**

We would like to thank N. Guiso and E. Njamkepo for sending us B. holsøesi strains. This work was supported by the Deutsche Forschungsgemeinschaft (SFB479-A2).

**REFERENCES**


