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New Virulence-Activated and Virulence-Repressed Genes Identified by Systematic Gene Inactivation and Generation of Transcriptional Fusions in *Bordetella pertussis*

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An in silico scan of the partially completed genome sequence of *Bordetella pertussis* and analyses of transcriptional fusions generated with a new integrational vector were used to identify new potential virulence genes. The genes encoding a putative siderophore receptor, adhesins, and an autotransporter protein appeared to be regulated in a manner similar to *Bordetella* virulence genes by the global virulence regulator BvgAS. In contrast, the gene encoding a putative intimin-like protein appeared to be repressed under conditions of virulence.

Bordetella pertussis is a strictly human pathogen responsible for whooping cough, an acute respiratory disease particularly severe in young children (18). It produces a number of toxins and adhesins involved in its pathogenicity. The coordinated expression of the virulence genes is controlled by the global sensor and regulator BvgAS (1). The genes under the positive control of BvgAS are called *vag* (for virulence-activated gene) genes. In response to environmental conditions, such as low temperature or the presence of $MgSO_4$ or nicotinic acid, *B. pertussis* undergoes a phenotypic modulation. The *vag* genes are downregulated, while another set of genes called *vrg* (for virulence-repressed gene) genes is upregulated (19).

We have scanned the *B. pertussis* sequences currently available on the Sanger Center website (www.sanger.ac.uk/Projects/B_pertussis) to identify new potential virulence genes (Table 1). We have developed a new suicide vector, pFUS2, for rapid gene inactivation by homologous recombination and generation of transcriptional fusions between the interrupted genes and promoterless *lacZ* (Fig. 1). pFUS2 was derived from pQE30 (Qiagen, Courtaboeuf, France) by (i) the replacement of the 960-bp *Eco*RI-blunted *Bgl*I fragment containing the *tac* promoter and the *bla* gene with a gentamicin resistance cassette, (ii) the insertion into the *Bam*HI and blunted *Eco*RI sites of the promoterless *lacZ* from pUTminiTn5lacZ2 (5) contained on a 3-kb *Bam*HI-blunted *Hin*dIII fragment, (iii) the insertion of a PCR-amplified 760-bp fragment carrying the RP4 origin of transfer from pJQ200mp18 (27) into the unique *Xba*I site, and (iv) the insertion into the *Cla*I and blunted *Bam*HI sites of a PCR-amplified 910-bp fragment containing the very 3′ end of *B. pertussis groES*, the intergenic *groES-groEL* region, and the 5′ end of *groEL* fused to the slightly truncated 5′ end of *lacZ*.

Gene inactivation with pFUS2 was first validated by targeting the known Bvg-activated genes *fhaB* and *ptx*, coding for the filamentous hemagglutinin (FHA) and pertussis toxin, respectively (21), and one Bvg-repressed gene, *vrg24* (19). Pairs of oligonucleotides were designed to amplify 350- to 550-bp internal fragments of the target genes by PCR. The amplicons were cloned into pFUS2 so that translation of these genes terminated at the *groES* stop codon, giving rise to transcriptional fusions with *lacZ*. Gene inactivations were performed in both *B. pertussis* Tohama I derivatives BPSM (bvg^+) (23) and BPLOW (Δ*bvg*). BPLOW carries a chromosomal deletion of *bvgAS* extending from the first *Eco*RI site upstream of *bvgA* to the first *Eco*RI site within *bvgS*. This strain was constructed by double homologous recombination using pSS1129 as described previously (28). Both *ptx* and *fhaB* fusions were expressed at high levels in BPSM and were modulated by $MgSO_4$ and nicotinic acid (Table 2). The expression of *ptx′-′lacZ* was very low in BPLOW, and that of *fhaB′-′lacZ* was undetectable. The expression of the *vrg24′-′lacZ* fusion in BPSM increased 2.5-fold in the presence of the modulators, and it was slightly higher in the Δ*bvg* background than in the bvg^+ background. Altogether, these results confirm the usefulness of pFUS2 to identify *vag* genes as well as *vrg* genes. Therefore, we used this vector to target genes encoding new potential virulence factors (Table 1).

Adhesins. Two genes code for putative proteins homologous to FHA. One of them, called *fhaL* (for FHA-like, large), corresponds to the largest open reading frame (ORF) of the entire genome. The gene encoding the other FHA-like protein, named FhaS (for FHA-like, small), harbors two frameshifts, and its 5′ region is not included in the current contig. Both genes appeared to be well expressed, albeit at much lower levels than *fhaB* (Table 2). Interestingly, both are regulated by Bvg and thus qualify as *vag* genes. The reasons for this apparent redundancy are not clear. FHA is of great importance to *B. pertussis* pathogenicity. Therefore, the bacterium may maintain backup gene copies. Alternatively, the poorly expressed copies might act as reservoirs for homologous recombination with the master gene to generate antigenic diversity, similar to the pilin genes in *Neisseria* (16). It is also conceivable that the three proteins play related but distinct functions, similar to the antigen 85 complex of *Mycobacterium tuberculosis* (2). Alternatively, the bacterium progressively sheds obsolete copies.

The gene encoding a putative signal peptide-bearing protein homologous to a salivary streptococcal adhesin (13) was identified and called *adhS*. This gene is part of an operon also including genes encoding a permease and an ATPase, and the corresponding protein shares limited similarity with periplas-

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TABLE 1. Characteristics of putative *B. pertussis* proteins

Protein	Amino acid sequence[a]		Size (aa[b])	Closest homolog	GenBank accession no.	% Identity[c]
	N terminal	C terminal				
FhaL	MNKHCFKLVH	GASATSRLPP	4,196	FHA (*B. pertussis*)	P12255	10
FhaS	AATAILTEIT	PGGSPFSLHR	2,431	FHA (*B. pertussis*)	P12255	22
AdhS	MKSRILALAA	AQQLAAALRP	311	Adhesin B precursor (*Streptococcus parasanguinis*)	P31305	27
BilA	MNKNIYRVVW	LLLAPVNFPY	1,308	Intimin (*E. coli*)	AAD02815	13
BexB	VIGSLIFRET	RAMRRRLHTR	246	Transport protein KPSM (*E. coli*)	P24584	30
DrnB	MKYAVVLAAL	DPAAIQRQCG	246	Extracellular DNAse (*Aeromonas hydrophila*)	O31222	45
MetC1	MTDESRHIDT	TQALARAASA	397	β-Cystathionase (*B. avium*)	Q07703	79
MetC2	MKFDTLLTHG	DLDQALRRED	383	β-Cystathionase (*E. coli*)	P06721	40
BllY	MAENFQPWEN	MRRGVVKQVD	370	4-Hydroxyphenylpyruvate dioxygenase (*Pseudomonas* sp.)	P80064	54
NprB	MXXPTXKAAR	QAWTDVGVLT	343	Extracellular metalloprotease (*Erwinia carotovora*)	Q99132	51
ZnpB	VTAPVPLFTL	VRIRHLMAGA	421	Zinc metalloprotease (*Campylobacter jejuni*)	CAB72997	25
Phg	MKPTSILARL	SLNIGYAYRF	418	Phg protein (*B. pertussis*)	CAB38010	100
AidB	MVGRSCHRAG	TATAGFRMMF	903	Adhesin AidA-I precursor (*E. coli*)	BAA15189	13
SphB1	MPPPAVPPQL	QLSASLTYRY	931	SSP-h1 protease (*Serratia marcescens*)	BAA33455	24
SphB2	MPSCWAWTAA	TASVVLRWRF	989	SSP-h1 protease (*S. marcescens*)	BAA33455	19
SphB3	MAQASARGMG	TGSLYLKVRF	1,076	SSP-h1 protease (*S. marcescens*)	BAA33455	14
BfrD	MKFYSSHPMP	SAMLTFKLSY	743	Hydroxamate ferrisiderophore receptor (*Pseudomonas aeruginosa*)	AAC06215	37
BfrE	MEKPLKSLDS	AAVLSFNIKY	756	Hydroxamate ferrisiderophore receptor (*P. aeruginosa*)	AAC06215	40

[a] Predicted sequences of the putative proteins.
[b] aa, amino acids.
[c] Percentage of identity between the *B. pertussis* protein and its closest homolog.

mic components of ATP-binding cassette transporters. It is therefore unclear whether it should be classified as an adhesin. This gene was expressed at a very low level and apparently was not modulated.

A gene was identified coding for a protein whose closest homolog is the enteropathogenic *Escherichia coli* intimin, and it was therefore called *bilA* (for *Bordetella* intimin-like). However, this putative protein is somewhat longer than intimin, and

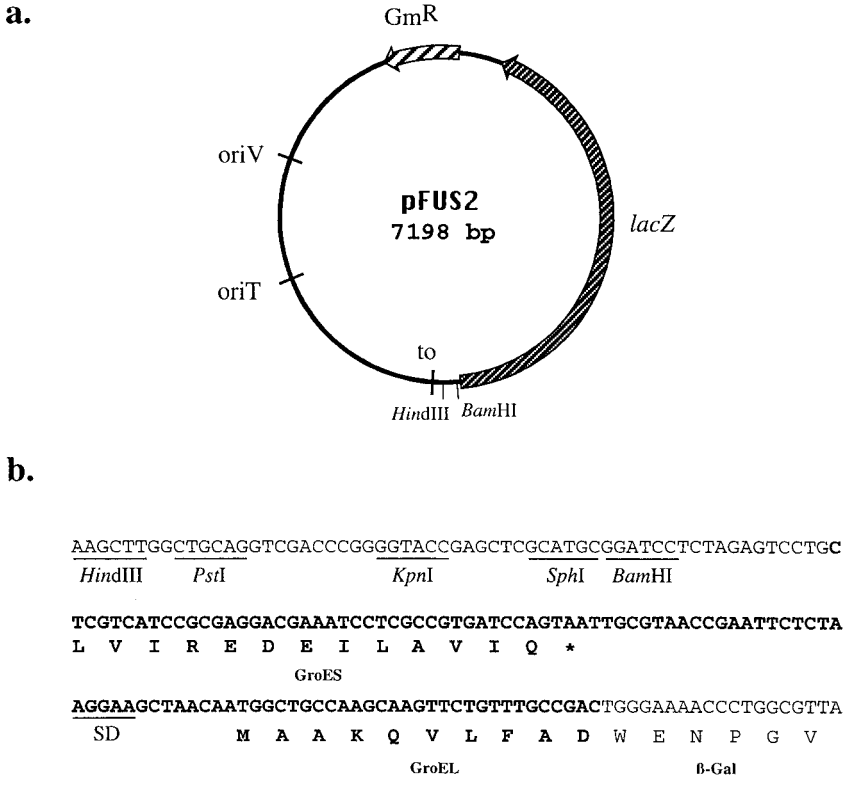

FIG. 1. Map of pFUS2. (a) Main features of the vector. Gm^R, gentamicin resistance cassette; oriT, RP4 origin of transfer, oriV, ColE1 origin of replication; to, a transcriptional terminator to prevent transcription initiation from vector sequences. (b) Sequence surrounding the multiple cloning site of pFUS2. The portion of the nucleotide sequence corresponding to the 3′ end of *groES*, the intergenic *groES-groEL* region, and the 5′ end of *groEL* is shown in bold. The 3′ end of *groES* and the 5′ end of *groEL* have been translated (in bold), as has the beginning of *lacZ*. SD represents the putative ribosome binding site sequence of *groEL*. β-Gal, β-galactosidase.

TABLE 2. β-Galactosidase activities of *B. pertussis* pFus2 integrants[a]

Target gene	Enzyme activity in integrant			
	BPSM with:			BPLOW with NA
	NA	Mg	Nic	
fhaB	5,094 ± 22	473 ± 68	2,133 ± 324	0
ptx	9,220 ± 2,673	800 ± 311	1,096 ± 218	46 ± 22
vrg24	207 ± 15	490 ± 50	496 ± 60	295 ± 10
fhaL	161 ± 16	17.5 ± 3.2	6 ± 3	<1
fhaS	260 ± 62	54 ± 10.7	20.7 ± 4.5	15.6 ± 9
adhS	9 ± 2	10 ± 4.6	6 ± 0.7	<2
bilA	287 ± 17	1,920 ± 291	3,680 ± 145	1,080 ± 62
bexB	0	31.7 ± 2.4	<5	0
drnB	39 ± 3	31 ± 1	30 ± 5	32 ± 12
metC1	177 ± 24	240 ± 65	226 ± 14	211 ± 54
metC2	45 ± 17	93 ± 22	88 ± 8	67 ± 16
bllY	91 ± 18	129 ± 16	159 ± 21	28 ± 5.8
nprB	62 ± 8	116 ± 22	104 ± 4	70 ± 15
znpB	80 ± 12	132 ± 40	120 ± 30	61 ± 20
pgh	48 ± 13	32 ± 8	43 ± 16	39 ± 20
aidB	148 ± 56	150 ± 22	183 ± 27	138 ± 56
sphB1	1,063 ± 265	23 ± 3	93 ± 46	0
sphB2	0	0	0	<2
sphB3	<5	5 ± 2	<2	<4
bfrD	789 ± 308	10 ± 1	144 ± 36	60.5 ± 17
bfrE	13 ± 4	10.1 ± 1	9.3 ± 3.2	16.3 ± 1.4

[a] Three independent clones of each recombinant strain were grown for 3 days at 37°C on Bordet Gengou (Gibco) agar plates containing 10% sheep blood without (NA), or with the modulating agents $MgSO_4$ (50 mM) (Mg) or nicotinic acid (20 mM) (Nic). The cells were scraped from the plates and suspended in phosphate-buffered saline. The cell density was estimated by optical density measurements at 600 nm (OD_{600}). Then the cells were broken by passage through a French pressure cell. β-Galactosidase activities were determined by measuring the initial rates of *o*-nitrophenyl-β-D-galactopyranoside hydrolysis at 420 nm and calculated according to the following formula: (ΔA_{420}/minutes × 1,000)/OD_{600} × volume [in milliliters] of cell lysate in the reaction mixture). The results are given as means ± standard deviations.

it lacks the two disulfide-bonded cysteines shown to be essential for the binding activity of intimin (12). The expression of *bilA* was very high in the Δ*bvg* background and was dramatically upregulated by nicotinic acid and $MgSO_4$ in BPSM. These features indicate that *bilA* is a *vrg* gene. The roles of the *vrg* genes in *B. pertussis* infection remain mysterious (1, 22). Recent evidence suggests that these genes may be expressed upon entry into eukaryotic cells or at high bacterial cell densities (25). The product of *bilA* has been recently identified in *Bordetella bronchiseptica* and has been shown to be involved in colonization in a rabbit model (K. E. Stockbauer, B. Fuchslocher, J. F. Miller, and P. A. Cotter, Abstr. 100th Gen. Meet. Am. Soc. Microbiol., abstr. B-184, 2000). It will be interesting to determine whether the protein is involved in cytoskeleton rearrangements in the host cell, like intimin is (6).

Capsule. The *B. pertussis* genome contains a complete operon for the biosynthesis of a polysaccharide capsule. Earlier literature reports have suggested that the bacterium is capsulated (20). A gene in the 5′ region of this operon, named *bexB* (for *Bordetella* capsule export gene B) based on the usual nomenclature for other species, was targeted by pFUS2. No expression of *bexB* was detectable in either BPSM or BPLOW, but surprisingly a low but significant level of activity was observed in the presence of $MgSO_4$. It is possible that the appropriate environmental conditions have not been met for optimal gene expression.

Enzymes. An ORF was found which encodes a putative signal peptide-bearing protein homologous to secreted DNases of several pathogens (3). We called this gene *drnB* (for DNase of *Bordetella*). Its expression was rather low and was not influenced by modulation.

The *B. pertussis* genome analysis revealed two ORFs encoding homologs of β-cystathionase. These genes were named *metC1* and *metC2* based on the nomenclature for other species. The product of *metC1* is highly similar to *Bordetella avium* osteotoxin, which metabolizes cystine into an osteoblast-toxic molecule (14). *metC1* was expressed at a fairly high level but was not Bvg regulated. In contrast, *metC2* was upregulated twofold in the presence of $MgSO_4$ or nicotinic acid, and its expression was slightly higher in the Δ*bvg* background than in the bvg^+ background, reminiscent of *vrg24*. Therefore, *metC2* might be a *vrg* gene. The small amplitude of modulation may reflect the indirect effect of modulating agents on *vrg* expression in *B. pertussis*. Whereas the transcription of *vag* genes is directly activated by binding of the BvgA regulator to their promoter regions, that of the *vrg* genes is indirectly regulated by a Bvg-dependent repressor (24).

The sequence analysis also uncovered an ORF encoding a protein homologous to the *Legionella pneumophila* legiolysin, which in fact is a dioxygenase (17, 30). After inactivation of the *B. pertussis bllY* (for *Bordetella* legiolysin) gene, no strong modulation was observed. However, the β-galactosidase activity of that fusion in the Δ*bvg* background was lower than in the bvg^+ background.

The *B. pertussis* chromosome contains several genes coding for proteases other than housekeeping proteases. Two of them, called *nrpB* (for neutral protease of *Bordetella*) and *znpB* (for Zn protease of *Bordetella*) in agreement with the names given to homologs in other species, were selected for inactivation by pFUS2. Both were expressed but were not regulated by Bvg.

Autotransporters. The genes for several autotransporters, in addition to BrkA, Tcf, pertactin, and Vag-8 (4, 8, 9, 11), were also identified in the genome of *B. pertussis*. Five of them (*phg*, *aidB*, *sphB1* [for serine protease homolog of *Bordetella*], *sphB2*, and *sphB3*) were targeted with pFUS2. The expression levels of *sphB2* and *sphB3* were very low or undetectable, while *phg*, *aidB*, and *sphB1* were better expressed. In addition, *sphB1* was strongly activated by Bvg, making it a new *vag* gene.

Iron metabolism. A number of genes encoding potential siderophore and heme receptors were uncovered. In particular, an outer membrane protein of unknown function (26) was found to be homologous to TonB-dependent siderophore receptors of various bacterial species. Its gene is followed by a second gene, coding for a 56.7% identical protein. The two genes, called *bfrD* and *bfrE* (for *Bordetella* ferrisiderophore receptor), are separated by approximately 200 bp. Expression of *bfrE* was very low and did not appear to be Bvg regulated. In contrast, *bfrD* was expressed at a high level at a Bvg-dependent fashion, making it the first *vag* gene involved in iron acquisition in *Bordetella*. Interestingly, siderophore production is repressed by Bvg in certain *B. bronchiseptica* strains but not in *B. pertussis* (15). This divergent regulation may relate to considerable differences between the two species in the ability to survive outside of their hosts (1).

In conclusion, pFUS2 has allowed us to uncover several new Bvg-regulated genes. Undoubtedly, additional members of the Bvg regulon remain to be found. In fact, BvgAS appears to control various aspects of *Bordetella* physiology in addition to virulence, including the production of a cytochrome (7), a porin (10), and cell wall hydrolase(s) (29). Further characterization of the Bvg regulon will greatly benefit from the development of transcriptomic and proteomic tools.

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