UNIVERSITY OF ALBERTA

CHARACTERIZATION OF Bvg-REPRESSED MOLECULES OF *Bordetella pertussis*

BY

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF MEDICAL MICROBIOLOGY AND IMMUNOLOGY

EDMONTON, ALBERTA

FALL, 1998

ABSTRACT

The etiological agent of whooping cough, *Bordetella pertussis*, expresses a number of virulence determinants which contribute to pathogenesis. Furthermore, the bacterium possesses the ability to coordinately regulate the expression of its virulence determinants in response to environmental stimuli through use of the BvgASR regulatory system. In *B. pertussis* this switching phenomenon is known as phenotypic modulation. This regulation results in the reciprocal expression of Bvg-activated genes (*vags*) and Bvg-repressed genes (*vrgs*.). While the importance of *vag*-expressing (X-mode) bacteria in *B. pertussis* pathogenesis is well established, the role of *vrg*-expressing bacteria (C-mode) is unknown.

Although much is known about the proteins which exist in the *B. pertussis* X-mode, relatively little is known about the proteins produced in the C-mode. We used SDS-PAGE and IEF techniques to demonstrate the existence of at least twenty-two Bvg-repressed molecules which are increased in C-mode-like bacteria. In addition, a series of monoclonal Abs (MAbs) were developed which are specific for the surface of C-mode *B. pertussis*. Using immunological and protein techniques we characterized two of these antigens (Bvg-repressed antigen-a [vra-a] and Bvg-repressed antigen-b [vra-b]) as surface-exposed proteins. Vra-a is expressed only in *B. pertussis*, and not in the related species *B. parapertussis* and *B. bronchiseptica*. Vra-b is present in *B. parapertussis* and *B. bronchiseptica*, but is expressed at lower levels which are not regulated by

Bvg.

Further characterization of Bvg regulation demonstrated that the vras are subject to the BvgASR regulatory cascade. Morever, using transposon mutagenesis we discovered two putative regulatory loci, *ompR* and *acn*, required for the expression of the vras and *vrgs*. Analysis of mutants in the HeLa cell invasion model demonstrates that *ompR* mutants are severely impaired in their ability to invade and survive within human epithelial-like cells; however, their adherence to these eukaryotic cells is not significantly affected. These data suggest that other regulatory pathways affect the regulation of the Bvg-repressed regulon. Furthermore, proper expression of this regulon is implicated to be crucial for invasion and survival *of B. pertussis* in epithelial cells. The identification and characterization of Bvg-repressed molecules (and the genes which encode and regulate them) may help elucidate a physiological role for modulation of this obligate human pathogen.

This thesis is dedicated to my wife Jody; my parents Arthur and Bea; my siblings Daryl, Robert, Wendy, and Kerry; my grandmother Agnus Chester; and high school science teacher Mr. Fred Gainer who showed me how fun science can be.

"Things should be kept as simple as possible, but not simpler."

-Albert Einstein

ACKNOWLEDGMENTS

I wish to sincerely thank my supervisor Dr. Mark Peppler for his assistance, advice, enthusiasm, and patience (when necessary) during my stay in his laboratory. Moreover, I would like to thank him for his enthusiasm as a professor and project supervisor back when I was an undergraduate student; it was key in my decision to pursue graduate studies in medical microbiology.

Appreciation is also expressed to Dr. Glen Armstrong and to Dr. Dennis Kunimoto for their assistance and advice as members of my supervising committee, and to Dr. Mike Belosevic and Dr. William Goldman as my external thesis examiners. Thanks also to Glen for his excellent teaching and obvious enthusiasm for science while coordinating the microbial pathogenesis course MMID 505, and for not sending me to the gallows after a certain H_2O mishap in his lab. Dr. Belosevic also deserves mention for providing the interesting experimental parasitology course ZOOL 452. Dr. John Elliot deserves great thanks for providing the advanced cloning course MMID 516, and for the open door to his laboratory and office when extra help was needed. His enthusiasm, scientific curiosity, and knowledge changed the way in which I think about my experiments. Medical Laboratory Science instructors Terry Chelich, Pat Letandre, and others are thanked for their excellent tutelage and encouragement while I was an undergraduate student.

Fellow students Halah Alhussaini, Gina Broitman, Max Cummings, Carol Ewanowich, Yvon de Moissac, Deborah Hemmerling, Lisa Purdy, Christine Szymanski, Laurina Turcotte, Rosa Vanmaele, and others are all acknowledged for their advice, assistance, and encouragement. The technologists and technicians (the ones who make these laboratories work) Kate Agapsowicz, Sharee Kuny, George Mulvey, Christina Rogers, Sann Vihn, Imtiez Zainul, and others are all thanked for their invaluable assistance and support.

I would also like to thank Richard Sherburne for his technical expertise in electronmicroscopy experiments and photography, and Deb McFarlane for her work in generation of transposon mutants.

Dr. Duncan Maskell, Dr. Tod Merkel, Dr. Alison Weiss, and many others all deserve thanks for their gifts of strains, reagents, protocols, and ideas.

My wife Jody, my family, and my extended family (the Stephens) are all thanked for their crucial encouragement and support.

Last, but not least, I wish to thank the Alberta Heritage Foundation for Medical Research and the Canadian Bacterial Disease Network for their generous financial support.

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2DGE	two-dimensional gel electrophoresis
Ab	antibody
Acn	aconitase
AIDS	acquired immune deficiency syndrome
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BGA	Bordet-Gengou agar
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin
bvg	*Bordetella* virulence gene
BvgR	*Bordetella* virulence gene repressor
CFU	colony forming unit
CTAB	hexadecyltrimethlyl ammonium bromide
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid}
ELISA	enzyme-linked immunosorbant assay
FBS	fetal bovine serum
FHA	filamentous hemagglutinin

Fur	ferric-uptake repressor
GTP	guanosine triphosphate
GFP	green fluorescent protein
IEF	isoelectric focusing
IRE	iron repressive element
IVET	*in vitro* expression technology
kb	kilobases
kDa	kiloDaltons
L-agar	Luria agar
L-broth	Luria broth
LOS	lipooligosaccharide
MAb	monoclonal antibody
MEM	minimal essential media
mRNA	messenger ribonucleic acid
MW	molecular weight
OmpR	outermembrane protein regulator
OMV	outermembrane vesicles
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PFGE	pulsed-field gel electrophoresis
PhoA	alkaline phosphatase
RNA	ribonucleic acid
RT	room temperature

SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulfate
SE	SDS-EDTA buffer
SSA	Stainer-Scholte agar
SSB	Stainer-Scholte broth
TBS	Tris buffered saline
TCT	tracheal cytotoxin
TE	Tris-EDTA buffer
TGS	Tris-glutamate saline buffer
vag	Bvg-activated gene
Vra	Bvg-repressed antigen
vrg	Bvg-repressed gene
WC	whole cell

Chapter I

INTRODUCTION

A. INTRODUCTION AND RATIONALE FOR EXPERIMENTATION

Bordetella pertussis, the etiological agent of whooping cough, is estimated to infect more than five million people worldwide and to cause approximately five hundred thousand deaths annually (54). *Bordetella pertussis* was originally described in 1906 by Bordet and Gengou as a member of the genus *Haemophilus* (17), and received its current genus classification in 1952 (88). It is a small fastidious Gram-negative aerobic coccobacilli that is closely related to other members of the genus *Bordetella*, *Bordetella parapertussis* and *Bordetella bronchiseptica*, which also cause upper respiratory infections in mammals (47, 54, 89). *Bordetella pertussis*, however, appears to be an obligate human pathogen with no animal or environmental reservoir being described.

Bordetella parapertussis also causes whooping cough in humans, although with lower frequency, and symptoms that are generally less severe (95). Although *B. parapertussis* has been isolated from sheep, these isolates appear to be phylogenetically distinct (127). *Bordetella bronchiseptica* differs from *B. pertussis* and *B. parapertussis*, infecting a number of mammalian species causing a variety of veterinary diseases which have a broad scope of severity and presentations (45). *B. bronchiseptica* can also infect immunocompromised

humans and clinically has become much more prevalent since the emergence of AIDS (70). Physiologically, *B. bronchiseptica* also differs from *B. pertussis* and *B. parapertussis* as a much less fastidious, motile bacterium that potentially can survive in environments outside of its mammalian hosts (96, 97).

Classical whooping cough, as caused by *B. pertussis*, is wide-spread throughout the world despite vaccination programs and antibiotic treatment that have reduced the number and severity of the cases in many countries. Classical whooping cough presents in three stages: the catarrhal, the paroxysmal and the convalescent stages. The catarrhal stage is characterized by nonspecific respiratory symptoms and general malaise that last about 7-10 days. The paroxysmal stage is characterized by vomiting and paroxysmal coughing episodes that can last several weeks before they eventually diminish as the host enters the convalescent stage.

The clinical presentation of classical whooping cough is most common in nonimmunized children, whereas in adults and vaccinated children the symptoms are generally less severe and are described as atypical or mild pertussis. Investigations of mild or asymptomatic pertussis have produced a variety of results and the existence of a true carrier state has been controversial (25). As stated, *B. pertussis* is nutritionally fastidious and a believed to be an obligate human pathogen with no environmental or animal reservoir. Hence, it is reasonable to speculate that such a carrier state does indeed exist, but that numbers and the location of the bacterium within its host have made it difficult to observe. Whether a classical carrier state exists for pertussis or not, it is clear

that the bacterium spends much of its life within the environments of the human host. Therefore, *Bordetella pertussis* must be highly evolved to live within its host, cause the disease process while evading the host immune response, and survive to be transmitted to other hosts to ensure the success of the bacterial species.

B. pertussis is known to produce a number of virulence factors, including toxins, which allow establishment within the human host and production of clinical disease (130). This has lead to the theory that whooping cough is a toxicosis and that production of these factors are sufficient for establishment of the pathogen and production of disease (95). However, several other traits of the bacterium have been described which lead one to speculate that the life-cycle of the bacterium, and the disease process it causes, may be more complex than previously thought.

It is well established that *B. pertussis* evolved the specialized ability to enter and survive within a number of human cell types. Crawford and Fishel were the first to describe this phenomenon as outgrowth of *B. pertussis* from a number of *in vitro* epithelial-like tissue culture systems following antibiotic treatment (29). Subsequently, Cheers and Gray described survival of the bacterium within murine macrophages in a disease model during what they termed the "complaisant" phase of infection (24). Subsequent to these experiments, studies by Ewanowich *et al.*, and others, well established that *B. pertussis* has evolved the means to enter and survive within mammalian epithelial and immune-effector cell lines (32, 33, 39, 68, 105, 114). Although the

bacterium is not "classically invasive" as it does not breach the primary site of infection to reach deeper host tissues, it is speculated that this invasive ability may be important for the bacterium to evade host immune responses, and perhaps be important in persistence and carriage of the pathogen.

In addition to the ability to produce a number of virulence factors including toxins and adhesins, and the ability to invade eukaryotic cells, *B. pertussis* also has the genetic ability to switch between distinct phenotypic states (61, 69, 92). Indeed, the production of the virulent and invasive phenotype of the bacterium is subservient to the state the bacterium expresses (33, 130). In 1931, Leslie and Gardner were the first to describe that *B. pertussis* can exist in a number of phenotypic states (69). Based on colony morphology, hemolysis, and antigenic characteristics they described four derivative phases (I to IV) of the bacterium. Although the nomenclature, number, and exact characteristics of these states differed in subsequent investigations (63, 92, 94), the basic truism that the bacterium can exist as a number of phenotypic derivatives is now well established and is termed "phase variation".

In addition to metastable spontaneous phenotypic switches, the ability of *B. pertussis* to freely switch phenotypes in response to environmental conditions was first observed by Bordet and Sleeswyck in 1910 (18). This phenomenon was analyzed in detail by Lacey in 1960 who showed that by altering growth conditions in a number of ways (such as temperature variation and ionic composition) *Bordetella* species could exist in three distinct "modes" based on antigenic characteristics: X-mode (xanthic), I-mode (intermediate), and C-mode

(cyanic) (66). Subsequently, Pusztai and Joo, and others, further demonstrated the importance of environmental growth conditions on the phenotypic state *of B. pertussis* (64, 78, 100). This switching phenomenon was termed "antigenic modulation" by Lacey, and is also called "phenotypic modulation". Signals which are known to induce phenotypic modulation are low temperature, and high concentrations of sulfate anions or β-vitamin derivatives (57, 66, 79, 100). These signals are not believed to occur within the human host, and what signals (if any) produce modulation *in vivo* is not known.

Investigations at the molecular level have lead to a great deal of knowledge about the biochemical and genetic nature of the major virulence factors of *B. pertussis*. Moreover, these investigations lead to the identification of a large DNA operon called *vir* and now known as *bvg* (*Bordetella* virulence gene), required for the expression of most of the virulence factors, and for the ability to switch between the virulent (phase-I- or X-mode-like) phenotype to the avirulent (phase-III- or C-mode-like) phenotype (5, 118, 122, 133). This locus encodes a two-component regulatory system, BvgAS, which is homologous to a large family of prokaryotic environmental regulators (5, 82). In virulent bacteria in the absence of modulating agents, the BvgAS regulatory system is ultimately responsible for activating the transcription of the genes encoding the major virulence factors and autogenous activation of *bvgAS* (103, 109, 118, 122). Collectively, these genes are known as the Bvg-activated-genes or *vags*. In avirulent bacteria (with a nonfunctional *bvgAS* locus) or in the presence of

modulating agents, BvgAS is said to be "off" and it does not produce the signals that facilitate expression of the *vags*.

In addition to coordinate regulation of the *vags*, another set of genes were discovered that are regulated reciprocally to the *vags*. These Bvg-repressed genes (*vrgs*) are expressed maximally in the presence of modulating agents or when the *bvg* locus is inactivated (64). In contrast to the well characterized role of the *vags* and their protein products in virulence and pathogenesis, the function and significance of the *vrgs* in the life-cycle of *Bordetella pertussis* has not been established. However, one bacterial strain (SK6) with a transposon insertion in a *vrg* was shown to be significantly less virulent in a mouse model of infection (14). In addition, another study suggested that *B. pertussis* modulates within human macrophages from the *vag*-expressing X-mode to the *vrg*-expressing C-mode (74).

Collectively, the ability of *B. pertussis* to modulate between phenotypic states; the ability of the bacterium to invade and survive within eukaryotic cells; and the fact that *B. pertussis* appears to be an obligate human pathogen; beg the hypothesis that modulation occurs during entry or inside human cells during the disease process, and that the expression of the *vrgs* may be important for the uptake or survival of the bacterium inside eukaryotic cells. Other hypotheses of course exist; the most extreme of which is that the *vrgs* and their products represent an evolutionary vestigial arm and serve no purpose during the bacterium's life-cycle and no function within its environments (73). It is clear, however, that testing of any of these hypotheses requires a better understanding

of the *vrgs*, their gene products, and their regulation. This was the impetus for the experiments presented in this thesis. Moreover, the ultimate goal of investigating *B. pertussis* modulation and Bvg-repressed molecules is to gain a more detailed understanding of the survival strategies of this human pathogen.

B. THE BvgAS REGULATORY SYSTEM

1. BvgAS Regulated Products of *Bordetellae*

In *Bordetella pertussis* many gene products have been described which are Bvg-activated. The encoding genes include the pertussis toxin gene (*ptx*) (53), the filamentous hemagglutinin (*fhaB*) locus (87, 119), the pertactin gene (*prn*) (22), the dermonecrotic toxin gene (*dnt*) (128), the tracheal colonizing factor gene (*tcfA*) (38), fimbrial genes (*fim-2 fim-3*) (87, 134), the adenylate cyclase gene (*cyaA*) (67), a gene encoding a porin-like protein (*ompQ*) (36), a locus encoding a putative adhesin and conferring serum resistance (*brkAB*) (34), as well as several genes which encode uncharacterized factors (37, 64, 132). These genes encode all the known virulence factors of *B. pertussis* with the exception of tracheal cytotoxin (TCT).

Many of the major virulence factors are shared between *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*; and similarly their dependence on Bvg-activation is also common between the three species (73, 76, 86, 110). The notable exceptions are pertussis toxin (6) and tracheal colonizing factor (8). Although *B. bronchiseptica* and *B. parapertussis* contain genes for pertussis toxin expression, they are silent due to an accumulation of mutations in their promoter regions (6). In addition, the expression of the Bvg-activated repressor, BvgR, has only been reported in *B. pertussis* (80).

In contrast to the commonality of the Bvg-activated factors of the mammalian *Bordetella* species, the Bvg-repressed factors appear unique to each

species. In *B. bronchiseptica* flagella expression and motility are Bvg-repressed traits (3, 4). In contrast, *B. pertussis* and *B. parapertussis* are non-motile, although presence of the regulatory locus (*frl*) and a silent copy of flagellin structural gene (*flaA*) have been noted in *B. pertussis* (3). Similarly, siderophore expression is seen to be Bvg-repressed in some strains of *B. bronchiseptica* but not regulated by Bvg in *B. pertussis* (41). In addition, expression of urease is a Bvg-repressed trait of some *B. bronchiseptica* strains, while *B. pertussis* does not produce urease (although it may contain a cryptic urease gene) (77).

In *Bordetella pertussis*, five Bvg-repressed genes were discovered through the use of Tn*PhoA* mutagenesis (64). Based on limited sequence analysis, these genes do not encode known virulence factors (12, 14). Using Northern and Southern blotting techniques it was shown that only *B. pertussis* expresses transcript of one of the genes (*vrg-6*), even *though B. bronchiseptica* and *B. parapertussis* appear to contain copies of the encoding DNA (14).

The *bvgAS* loci of these species are themselves highly homologous, responsible for phase variation and phenotypic modulation, and furthermore these species respond to the same *in vitro* modulating signals (7, 73, 86, 110). The *bvg* loci of *B. pertussis* and *B. bronchiseptica* are 96% homologous at the DNA level, and are functionally interchangeable (7). At the protein level, the few amino acid differences between of *B. bronchiseptica* and *B. pertussis* occur mostly in the periplasmic domain of BvgS, while BvgA is identical between the species. These differences in BvgS are responsible for a greater sensitivity of

B. bronchiseptica to the *in vitro* modulating agents nicotinic acid and sulfate anions relative to most *B. pertussis* strains (73).

2. Phase Variation

Bvg was shown to be involved in phase variation by Wiess and Falkow by mapping and sequence analysis of a series of phase derivatives that were sequentially isolated from *B. pertussis* strain Tohama I (129). The phase switch to the avirulent (phase-III) phenotype is the result of a frameshift mutation by an insertion of a G residue in a string of six G residues in the coding sequence of *bvgS*, and backwards variation to the virulent (phase- I) phenotype occurs by reversion of this mutation (117). Interestingly, a similar mechanism exists for fimbrial phase variation in *B. pertussis* (87, 134). Some Tohama variants have a high rate of phase variation (10^{-3} to 10^{-4}) which has been reported to be due to a mutator phenotype (117, 118). The frequency of phase variation in *B. pertussis* strains ranges from 10^{-3} to 10^{-6} and is reversible at similar frequencies (93). Analysis of *B. bronchiseptica* has shown phase variation to be due to deletions in the coding sequence of *bvgS* resulting in the avirulent phenotype (7, 86). This variation is thus nonrevertable. Although the low frequency of phase variation raises doubts as to its biological relevance, it should be noted that clinical phase variants have been isolated from humans with increasing frequency during the course of infection (62).

3. Phenotypic Modulation

The similarity between the phenotypes of *bvg* phase variants and *Bordetellae* that were phenotypically modulated by environmental conditions, combined with the homology of BvgAS with environmental regulators suggested that Bvg was involved in the process of phenotypic modulation. The first experimental indications that Bvg was responsible for modulation came from experiments which analyzed the function of BvgAS in *E. coli*. *E. coli* harboring plasmids containing *bvg* and the contiguous *vag* locus *fha* were shown to express proteins which react with FHA-specific antibodies (119). This phenotype is dependent on a functional *bvg* locus and furthermore is modulated by the same environmental signals as in *B. pertussis*. Subsequent studies using *fhaB* gene fusions demonstrated that *bvg* acts in *trans* (83), and that overexpression of BvgA in the absence of BvgS is sufficient for expression of *fhaB* (102). Moreover, the expression in the presence of overexpressed BvgA is not subservient to environmental modulators.

Direct sensing of environmental conditions by *bvg* was established by analysis of mutants which expressed *vag* loci, but were no longer responsive to environmental regulators (64, 81). The mutations were found to alter BvgS at cytoplasmic regions just downstream of the transmembrane region in a domain known as the linker (81). These mutations delineate the gene activating function of *bvg* from its environmental sensing function and demonstrate BvgS to be the sensor protein.

Recent work in *B. bronchiseptica* has revealed an intermediate phase of modulation mediated by *bvg* which corresponds to the I-mode of *B. pertussis* first described by Lacey in 1960 (28). This phenotype (Bvgi) does not express a subset of the Bvg+ factors while expressing a new set of I-phase specific factors. Genetically this phenotype is locked in a mutant in which there is an amino acid substitution near the primary site of phosphorylation in BvgS. This phenotype is also expressed in wild-type strains in response to concentrations of environmental modulators which are less than is required for modulation to the C-mode and thus named "semi-modulating" conditions. This I-phase may represent an important transitional phenotype between the X- and C-modes of *Bordetella* and may be important in the complex life-cycles lived by *Bordetellae* in their environments

4. Domain Structure of BvgA and BvgS

BvgA is a typical small (209 amino acid; 23-kDa) cytoplasmic response regulator with a C-terminal helix-turn-helix DNA binding domain, and a N-terminal receiver domain which contains conserved amino acid residues (including D-54) common to this family of proteins (5, 120, 122). BvgS is a large (1233 amino acid; 134-kDa) transcytoplasmic membrane sensor with a large N-terminal periplasmic region and cytoplasmic linker and transmitter domains. However, BvgS is distinguished from many sensor proteins by having an additional receiver and C-terminal domain which follow the transmitter domain. Based on primary sequence homologies with other response regulators it is predicted that the

unique N-terminal periplasmic region of BvgS senses specific environmental changes resulting in a signal that is relayed to the transmitter domain which has both a typical conserved ATP binding site and a conserved histidine (H-729). The common model for such two-component regulatory systems is that the sensor (BvgS) senses an environmental stimulus via its periplasmic domain and this signal is relayed via phosphotransfer (following autophosphorylation) from the conserved histidine of the cytoplasmic transmitter domain to the conserved aspartate of the N-terminal receiver domain of the cytoplasmic response regulator BvgA. This basic model then predicts that the phosphorylation state of the regulator alters its properties to affect transcription of specific genes (82, 122).

The additional receiver and cytoplasmic domains of BvgS are separated from the transmitter and each other by alanine/proline rich regions, and both domains contain amino acids which are conserved among two-component systems (including D-1023 of the receiver, and H-1172 of the C-terminal domain). The domain organization of BvgAS is presented in Figure I.1. These additional domains suggest that the true mechanism of signal transduction via BvgS is more complex than the basic model proposes. Much recent research has demonstrated the mechanistic complexity of this signal transduction system, and illustrates that BvgAS contains a complex network of potential signal input and output switches which may be important in the fine global regulation of the regulons by the *bvg* virulence control system (122, 124).

5. Model of the BvgAS Phosphorelay Cascade

The current model of the BvgAS phosphorelay cascade adheres to the basic model of phosphotransfer from the transmitter of BvgS to the receiver of BvgA, while demonstrating the absolute requirement for the BvgS receiver and C-terminal domains in the process (15, 16, 121, 122, 124, 125). After autophosphorylation of H-729 of the transmitter, phosphotransfer occurs to the D-1023 residue of the reciever. Following phosphorylation of D-1023, the H-1172 residue of the C-terminus is phosphorylated and mediates the phosphorylation of D-54 of the BvgA receiver (124). BvgS is believed to act as a dimer based on *trans* dominant negative mutations of BvgS, and allelic complementation of certain combinations of BvgS mutations (15, 16, 118).

Although the receiver is sufficient for BvgS autophosphorylation, critical for dephosphorylation of transmitter and C-terminal domains, and essential for phosphotransfer from transmitter to the C-terminus; it is currently unclear if the receiver of BvgS acts as a phosphodonor for the C-terminus or whether the transmitter acts as the phosphodonor. The latter mechanism has been proposed in a model whereby the phosphorylation of the receiver results in rephosphorylation of the transmitter and subsequent phosphotransfer to the C-terminus (124). Regardless of the mechanisms of phosphotransfer to the C-terminus, the requirement of the BvgS receiver and C-terminal domains is believed to be important in the fine regulation of its environmental response, and perhaps function in reception of alternate signal inputs.

6. Regulation of Transcription by BvgA

Analysis of BvgA deletion mutants demonstrated that it is required for expression of the *bvg*-regulated virulence determinants in *B. pertussis* (102, 120). Furthermore, overexpression of BvgA is sufficient for expression of *fhaB* in *E. coli* in a modulation independent manner (102). Work by Boucher *et al.* separated the functions of BvgA through deletion analysis (19). Deletions in the C-terminal domain of BvgA abolished DNA binding and transactivation of gene expression. BvgA with deletions in the N-terminus could still bind specific DNA sequences but did not transactivate, thus confirming the N-terminus to be the activating domain. In addition, phosphorylation of BvgA was seen to enhance binding of target DNA *in vitro.*

cis-acting sequences important for activation of *bvg*-activated genes have been analyzed with differing results for different *vags.* The *fhaB* and *bvg* loci are closely linked and transcribed from divergent promoters located between their coding sequences (103, 109, 119). In bacteria with inactive BvgAS, only the relatively weak *bvg* P2 promoter is active, and its activity likely accounts for basal levels of *bvg* transcript and BvgA and BvgS proteins. In conditions with active BvgAS, transcription occurs from the P1, P3, and P4 promoters of *bvg* and from the divergent *fhaB* promoter. In this state transcription from P2 is precluded presumably because binding of phosphorylated BvgA impedes binding to P2 which overlaps the site of the bound BvgA complex. P1 is a strong promoter and this reasonably accounts for the increased levels of *bvg* transcript in bacteria with active BvgAS. While the P1, P2, P3 promoters produce *bvg* transcript the P4

promoter produces an antisense RNA which initiates just upstream of *bvg*, and its role (if any) in regulation of transcription is unexplored.

Evidence suggests the DNA sequences that BvgA binds appear as inverted repeats upstream of the *fhaB* promoter and as direct repeats upstream of the *bvg* promoter (101). Indeed, the presence of these sequences contained on a plasmid in *E coli* reduced the expression of a *fhaB* reporter presumably because of titration of the BvgA activator (84). Comparison of these sites reveals the consensus binding sequence of the repeats to be the heptanucleotide sequence TTTCCTA. However, a more recent study has suggested that the true region of binding for phosphorylated BvgA to the *bvg* promoter encompasses a pair of inverted repeats just downstream of the direct repeats originally described (60).

Investigations of the binding sites for BvgA at the *cyaA* and *ptx* and other *vag* promoters have not revealed significant homology with the binding sites of the *fhaB* and *bvg* promoters (15, 21, 50, 52, 60, 72, 118, 134). However, data indicates that for all the promoters the presence of DNA repeats with particular spacings and position are important for the interaction of BvgA, and thus important for transactivation. Several lines of evidence also indicate that oligomerization of BvgA at the *cis*-activating sequences may be important in regulation (19, 20, 59, 72). Furthermore, phosphorylation of BvgA has been shown to be critical for effective binding of target DNA sequences (19-21, 59, 135).

7. Differential Regulation by BvgAS

One of the intriguing aspects of *vag* expression was the early observation that although BvgAS mediated *fhaB* and *bvg* regulation and expression could be reconstituted in *E. coli*, expression of other *vags* such as *cyaA* and *ptx* could not (83). These observations lead to the theory that an accessory factor is important for the expression of these differentially expressed *vags* (30, 56). Alternatively, DNA topology has been reported to be important for expression of the *ptx* promoter in *E. coli*; however, *ptx* expression in these experiments was not subject to environmental modulation (108).

In addition, these *vags* show a different time-course of activation or repression than *bvg* in response to environmental conditions (98, 107, 111). Activation of *fhaB*, and *bvg* promoters occurs quickly (within minutes) while activation of the *cyaA* and *ptx* promoters takes much longer (after hours) (98, 111). These temporal differences have been correlated to the concentration of BvgA (98). Conversely, repression of *ptx* and *cyaA* occurs within minutes while repression of the *bvg* promoters occurs hours later (98, 107). It has been reported that during this repressive switch the amount of total BvgA does not decrease, and thus one can speculate that the decrease in *vag* expression is due to a decrease in the amount of phosphorylated BvgA (98). As previously stated, the phosphorylation state of BvgA is critical for binding and transactivation of the *vag* promoters, and thus its concentration is likely involved in the differential regulation of *vags*.

The search for accessory factors involved in the expression of differentially regulated *vags* by mutational screening have been unsuccessful. Mutations which specifically reduce the expression of *cyaA* and *ptx* in *B. pertussis* were seen to alter the C-terminal domain of BvgA or the levels of the α-subunit of RNA polymerase (23, 116). Therefore, these mutations do not reveal accessory factors, but rather demonstrate that there are differences in the transcriptional complex or the binding of BvgA at these promoters which may be important for the differential regulation seen. However, one factor (Bvg accessory factor or Baf) has been identified via cloning by virtue of its ability to activate a *ptx* reporter in the presence of *bvgAS* in *E. coli* (30).

Recent studies have demonstrated that *E. coli* can express *ptx* via *bvgAS* transactivation when the bacterium is grown in minimal media (123). In addition, phosphorylated BvgA was seen to be sufficient for efficient *in vitro* transcriptional activation of *cyaA*, and *ptx* promoters but only in the presence of *B. pertussis* RNA polymerase and not *E. coli* RNA polymerase (115). Thus, it appears the bvgAS locus is sufficient for regulation of the *vag* loci but that differences in the interactions of BvgA and RNA polymerase in forming transcriptional complexes at their promoters may result in the differential regulation by BvgAS of the different classes of *vags.* In addition, other accessory molecules such as Baf may augment transactivation of certain genes. Currently, the molecular details of such interaction are matters of much investigation. What is clear presently is that the BvgAS system possesses many intricacies in its environmental sensing and

gene activation mechanisms. Furthermore, these intricacies may be important in the delicate regulation of virulence by *Bordetella pertussis in vivo.*

8. *vrg* Regulation In *Bordetella*

Using Tn*PhoA* mutagenesis, Knapp and Mekalanos first described the genes of the *vrg* regulon of *B. pertussis* which are expressed reciprocally to the major virulence factors encoded by the *vag* regulon (64). Sequence analysis of the 5' regions of the *vrgs* revealed an intragenic consensus region for four of the five *vrgs* (12). Furthermore, this intragenic region was shown to be sufficient for *bvg*-repressed gene expression (12, 13). Southwestern blots using sequences containing this region revealed binding of a 34-kDa protein which was bound less under modulating conditions (13). Hence, the binding of a *vag* product which acts as a transcriptional repressor of the *vrgs* was proposed to be the mechanism for *vrg* regulation for at least four *vrgs* . Using Tn*5* mutagenesis, Merkel and Stibitz identified five mutants with a phenotype constant with the mutagenesis of the putative repressor (80). All of these mutations mapped downstream of the *bvgAS* locus and resulted in constitutive expression of a *vrg* reporter fusion while demonstrating normal *vag* regulation. Moreover, precise mutations of this locus demonstrated the same phenotype, thus showing this phenotype was not due to polar effects of Tn*5*. Constitutive expression of the *vrg* which does not display a consensus region (*vrg-73*) was also seen in these mutants showing this *vrg* to be subject to regulation by the repressor as well.

Hence, the *vag* encoded repressor, BvgR, is involved in Bvg-dependent regulation of all the *B. pertussis vrgs* described currently.

In *B. bronchiseptica*, flagella expression has been shown to be Bvg-repressed (4). Analysis of *bvg*-repression of flagella has revealed that BvgA directly represses the master regulatory locus of flagella production *frlAB* and the structural flagellin gene *flaA* (3). *frlAB* expression controls a regulatory cascade which is necessary for the expression of the complex flagellar synthesis machinery. Hence, in *B. bronchiseptica* Bvg-repression has been shown to be due to repression of a transcriptional activator as opposed to the activation of a transcriptional repressor in *B. pertussis*. BvgR-dependent regulation of *vrgs* have not been described for *B. bronchipseptica* implying that the mechanisms for *vrg* regulation may be fundamentally different between *B. pertussis* and *B. bronchiseptica*. A schematic for BvgAS global regulation is presented in Figure I.2.

C. OTHER REGULATORY SYSTEMS

Although the BvgAS regulatory system is the best characterized regulatory system of *B. pertussis* and paramount in the expression of its virulent phenotype, other factors have been described which are likely important in the life-cycle of *B. pertussis*. These include the *baf* gene (30), the *btr* gene (9), the *fur* gene (11), the *tex* gene (40), and several histone-like proteins (48, 49, 106). The *btr* gene encodes a protein with homology to FNR-like transcriptional activator family

which act under conditions of low oxygen tension or oxidative stress (9). Its role in the virulence of the aerobe *B. pertussis* is unknown. *B. pertussis* produces siderophore and specific membrane proteins in response to iron-limiting conditions (1). The ferric iron uptake regulator gene (*fur*) of *B. pertussis* has been cloned and based on analogy to other systems is likely critical for the response to iron-limiting conditions (11). Initial studies indicate that *fur* is important for iron acquisition in *Bordetellae* (10, 42, 51, 58), but its importance in virulence is unexplored. The *tex* (toxin expression) gene encodes a transcriptional accessory protein with significant homology to the mannitol repressor protein (40). Preliminary studies indicate it has a role in the expression of *ptx* and *cyaA*. In addition, several histone-like molecules have been identified in *B. pertussis* (48, 49, 106). In other organisms these DNA binding proteins affect DNA topology and regulate expression of many genes, including ones which are important in virulence (35). Although these factors have not yet been implicated in *B. pertussis* virulence, they and other regulatory systems may be important for *B. pertussis*. The importance of multiple regulatory systems which sense multiple signals is well established in the virulence of many bacteria, and seems to be the rule rather than the exception (35). Hence, the investigation of such systems may prove illuminating in the understanding of pathogenic mechanisms in *B. pertussis*.

D. VIRULENCE REGULATION AND PATHOGENESIS

1. *Bordetella pertussis*

As previously stated, *B. pertussis* produces an array of putative virulence factors, most of which are regulated by the BvgAS machinery. These include adhesion molecules: filamentous hemagglutinin (FHA), pertactin, and fimbriae; and toxins: dermonecrotic toxin, pertussis toxin, and bifunctional adenylate cyclase toxin/hemolysin. The adhesins are involved in bacterial adherence to a variety of cell-lines, and their apparent redundancy and possible cooperative effects demonstrate the importance of effective adhesion in pathogenesis (87, 100). The adhesins, FHA and pertactin, are also important in the invasion of epithelial-like cells (32, 33, 68).

The toxins of *B. pertussis* are believed to result in most of the clinical pathogenesis seen in whooping cough. Adenylate cyclase acts by elevating the concentration of cyclic AMP in eukaryotic cells to a supraphysiologic concentration (55). Intoxication of host immune-effector cells is believed to be protective for the bacterium in response to the host immune response. Pertussis toxin is a member of the A-B toxin family whose A-subunit, S1, catalyzes ADP-ribosylation of GTP-binding proteins after delivery of the toxin by eukaryotic cells by the B-subunit (126). This results in a toxic effect by the uncoupling of eukaryotic signal transduction pathways to their membrane signaling receptors. Pertussis toxin is believed to cause many of the systemic effects of whooping cough, and like adenylate cyclase toxin has many effects on host immune

systems. In stringent mouse models, both adenylate cyclase toxin and pertussis toxin have been shown to be critical for virulence and pathogenesis (46, 130). Dermonecrotic toxin induces actin reorganization, multinucleation, DNA synthesis, and membrane organelle proliferation in eukaryotic cells (65, 99, 113). Its effects, including vasoconstriction (31, 91), are believed to contribute to the clinical pathogenesis of whooping cough. Tracheal cytotoxin is a muramyl peptide derived from the peptidoglycan of the bacterial cell wall, and differs from the other virulence factors by not being Bvg-regulated (43, 44, 71). It causes cilliostasis and cell death in cells of the epithelium, and hence is implicated to interfere with the clearance of the bacterium.

The multiplicity of the virulence factors of *B. pertussis* may reflect the necessity *of B. pertussis* multiple cooperative factors which produce establishment and pathogenesis, while avoiding a host antibody response to any particular factor. Recent studies have demonstrated the importance of cell-mediated immunity in response to *B. pertussis* (85, 104). What role phenotypic modulation has in the generation or avoidance of cellular immunity is unknown. It should be noted that in comparison to other mucosal pathogens such as *Neisseria gonorrhoeae*, *B. pertussis* has a limited ability to genetically alter its surface proteins (35, 112). Hence, environmental modulation of the expression of virulence factors by the BvgAS regulatory system may confer critical regulated expression of virulence factors in response to temporal need, metabolic efficiency, and host responses. Such regulated expression by two-component

regulatory systems is known to be important for many pathogens in a variety of micro-environments.

The importance of such a response has been proposed for *B. pertussis* using Bvg from consideration of the differential regulation of Bvg-activated factors (111). The faster activation response of the adhesin FHA, in comparison to the expression of toxins (pertussis toxin and adenylate cyclase), has been speculated to be due to the need to express adhesins first to establish infection; and express toxins later to maintain infection, and cause pathogenesis. Moreover, timely expression of these virulence factors may avoid a successful host immune response.

In contrast, demonstration of the possible importance of a regulated Bvg-repressed response within the milieu of host microenvironments has remained elusive. One study has proposed that *B. pertussis* modulates within human macrophages to affect intracellular survival (74). However, the results and conclusions of a subsequent study by the same author contradicted these initial findings (75). In addition, a Bvg-repressed gene was seen to be required for full virulence in a mouse model (14). The gene, *vrg-6*, has limited homology to genes encoding adhesins and thus it has been hypothesized that its expression along with other *vrgs* may be important in some environmental niche within the human host. However, the mutant which was less virulent was created by a transposon mutation which might cause polar effects, and the host strain it was produced in is noted to be atypical of the species (90). Thus, these results must be viewed with caution. Furthermore, the results of virulence studies in all animal

models should be viewed with some skepticism until collaborating results show them to be relevant in the natural host.

2. *Bordetella bronchiseptica*

As stated, most of the Bvg-activated virulence factors are shared between *B. pertussis* and *B. bronchiseptica* (with the exception of pertussis toxin and tracheal colonizing factor), and they are accepted to be responsible for the majority of pathogenesis resulting from *B. bronchiseptica* infection. In contrast to *B. pertussis*, infection studies *in B. bronchiseptica* can be done in a relevant host species. Although there is some evidence that Bvg-repressed molecules may be important for intracellular tropism in *B. bronchiseptica* (8, 26, 77, 133), thorough *in vivo* studies indicate that the Bvg+ phenotype is sufficient for establishment of disease in animal hosts (2, 27). In contrast, Bvg- phase or Bvgi phase bacteria are much less virulent than Bvg+ parental strains (2, 27, 28) Moreover, in these studies no immunological evidence was found for modulation within the hosts, and ectopic expression of the Bvg- factor flagella impedes the establishment of infection (2, 27).

The ability of *B. bronchiseptica* to survive in nutritionally limiting environments, including those which would produce a modulating environment due to low temperature, has lead to the hypothesis that the Bvg- phenotype is important in survival outside of the mammalian host (28). Bvg- factors such as flagella and siderophore have been speculated to be important for survival of the bacterium in such an environment. Furthermore, the environmental switching of

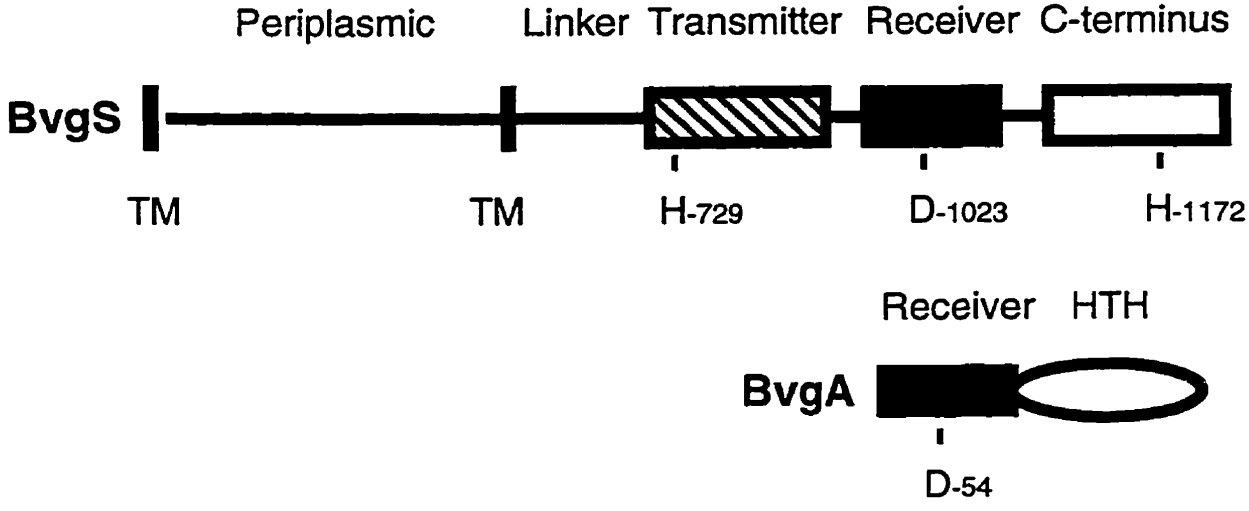
phenotypes has been speculated to be important in transmission of *B. bronchiseptica*. Although the somewhat higher sensitivity of *B. bronchiseptica* to *in vitro* modulators relative to *B. pertussis* has lead to the hypothesis that modulation is only significant in *B. bronchipseptica* (73), these signals are not believed to be relevant for *B. pertussis*. Hence, the elucidation of the role of modulation in *B. pertussis* requires much further consideration and study.

E. CONCLUSIONS

It is clear that *Bordetella* species have evolved a complex repertoire of factors involved in virulence and pathogenesis, and furthermore have evolved the means to regulate these factors in response to environmental conditions. What is much less clear is what role this regulation has in the vitality of the human pathogen *B. pertussis*. The ability of *B. pertussis* to establish infection, produce pathogenesis, avoid a host immune response, and survive to be transmitted to new hosts are complex behaviors encoded by the genes of many virulence factors and regulated by the *bvg* operon. It is apparent that these abilities are the result of a complex machinery to which the bacterium dedicates a great deal of its genome, and a significant amount of its metabolic energy. The ability to phenotypically modulate is inherent to this virulence machinery and can thus can be assumed to be relevant in the life-cycle of this human pathogen. Furthermore, the reciprocal nature of the expression of Bvg regulons suggests

that the *vrgs*, in addition to the *vags*, may have relevance for *B. pertussis*. Hence, studies of the Bvg-repressed genes, their products, and their regulation may further refine our understanding of the complex life-cycle of this intriguing bacterium.

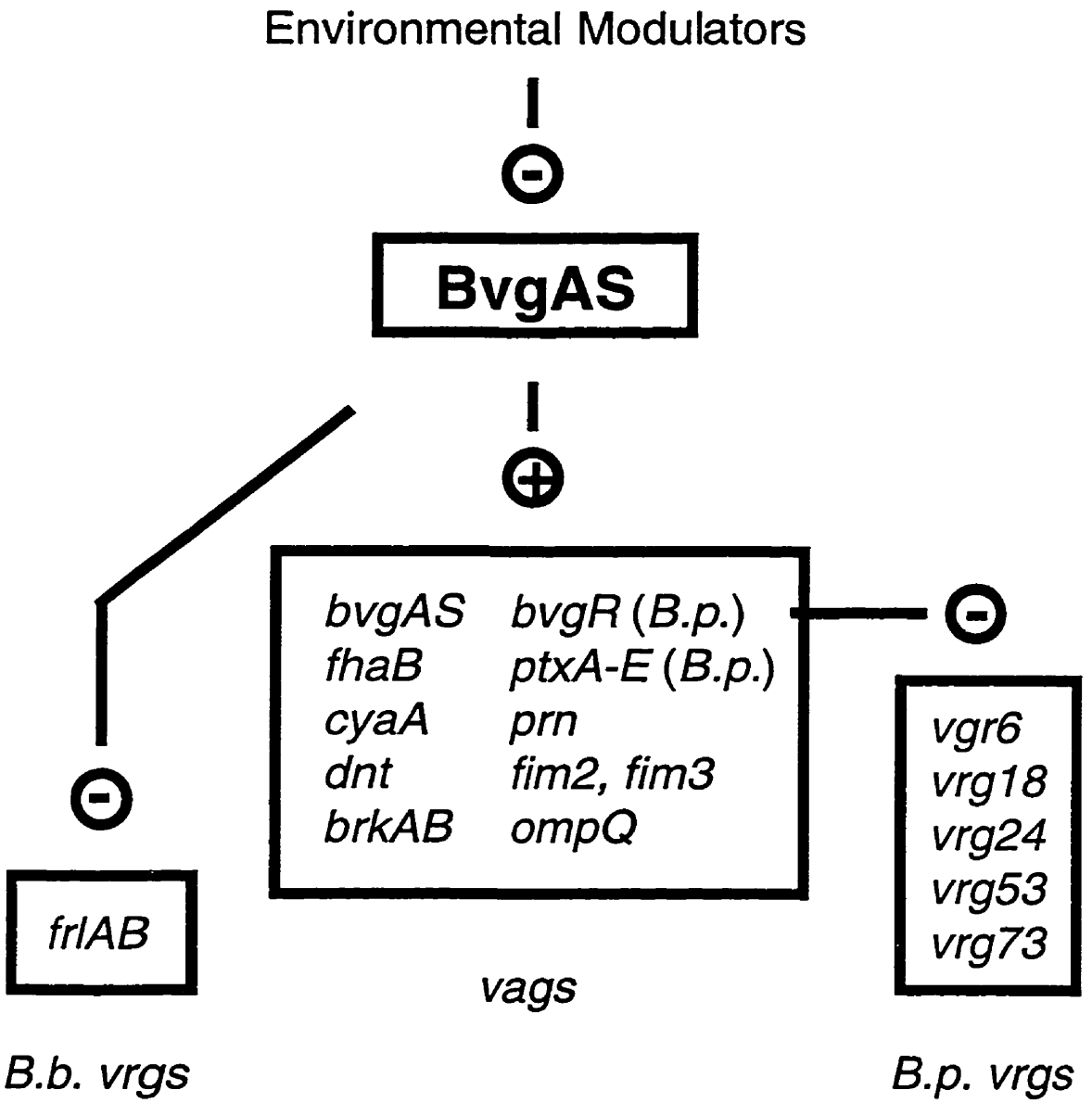
Figure I.1. Domain Organization of BvgAS[1]



[1]Selected features of the BvgA and BvgS proteins including sites (single letter amino acid code) of phosphorylation relavent in the model of the Bvg phosphorelay cascade. Transmembrane sequences (TM) and helix-turn-helix (HTH) motifs are indicated.

Adapted from: **Uhl, M. A., and J. F. Miller.** 1994. *Bordetella petussis* BvgAS virulence control system, p. 333-349. *In* J. A. Hoch and T. J. Silhavy (eds.), Two-Component Signal Transduction. American Society for Microbiology, Washington, DC.

Figure I.2. Bvg Globulon[1]



[1]Schematic representation of selected features of the Bvg global regulons of *B. pertussis* (*B.p.*) and *B. bronchiseptica* (*B.b.*). The *vags* shown are all shared between *B. pertussis* and *B. bronchiseptica* expect *bvgR* and *ptx*. The master regulator of *B. bronchiseptica* flagella expression (the *vrg* locus *frlAB*) is directly repressed by BvgAS while repression of the *B. pertussis vrgs* involves *B. pertussis* BvgR.

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Chapter II

IDENTIFICATION OF TWO Bvg-REPRESSED SURFACE PROTEINS OF *Bordetella pertussis*[1]

A. INTRODUCTION

Bordetella pertussis, the etiological agent of whooping cough, is known to possess an array of virulence factors which contribute to pathogenesis (49). These include pertussis toxin, filamentous hemagglutinin, and the bifunctional adenylate cyclase toxin-hemolysin. The coordinate expression of virulence factors in *Bordetella* species is regulated by the *bvg* locus (formerly known as *vir*) (5, 41, 44, 45, 50). The *bvg* locus is comprised of the *bvgA* and *bvgS* genes which encode a cytoplasmic transcriptional activator and a transmembrane sensor protein, respectively (39, 44, 46). These proteins are members of a large family of two-component bacterial response regulators common to a number of pathogens (5, 33). In *B. pertussis*, stimulation of the BvgS sensor protein results in a site-specific autophosphorylation and phosphotransfer cascade which ultimately results in phosphorylation of the BvgA activator (13, 47). In this state, the active BvgA molecule increases the transcription of the *bvg* locus (40), and a set of genes known as the *vir*-activated genes (*vags*) (20, 27, 51). These include

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[1] A version of this chapter has been published. **Stenson, T. H., and M. S. Peppler.** 1995. Infect. Immun. **63**:3780-3789

the pertussis toxin gene (*ptx*) (24), the filamentous hemagglutinin (*fhaB*) locus (45), the pertactin gene (*prn*) (14), fimbrial genes (*fim-1 fim-2*, *fimX*) (44, 52), the adenylate cyclase gene (*cyaA*) (30), a gene encoding a porin-like protein (*ompQ*) (19), a locus encoding serum resistance (*brkAB*) (18), as well as several genes which encode uncharacterized factors (20, 27, 51). In the case of the *bvg* locus and the *fhaB* gene, direct activation by binding of the BvgA protein to the promoter region of target genes has been shown to be the mechanism for transcriptional activation (23, 39-41, 44, 45). For other genes direct binding of BvgA or homologous protein binding sites in the promoter DNA have not been shown (23, 44). This indicates that their regulation by *bvg* may somehow differ from the *bvg* regulation of the *fhaB* locus and the autogenous regulation of *bvg*.

The control of virulence determinants is seen in two different phenomena: phase variation and antigenic modulation. In *Bordetellae* virulence regulation, phase variation describes an event wherein changes in the DNA coding sequence results in an inactive BvgS protein, and thereby a switch from the virulent phenotype (phase-I) to the avirulent state (phase-III) (34, 43). This switch occurs at a frequency of approximately 10^{-3} depending on the bacterial strain and is rarely reversible (a frequency of 10^{-6} or less). Antigenic modulation results from changes in the environment which are sensed by the BvgS protein (27, 32, 44). Such environmental factors as low temperature, and high concentrations of sulfate anions or β-vitamin derivatives result in the inactivation of the BvgS protein and the down-regulation of virulence gene expression,

thereby resulting in a switch from the virulent phenotype (X-mode) to the avirulent phenotype (C-mode) (26, 28, 31). The modes seen during antigenic modulation are likely homologous to the phases seen during phase variation; however, antigenic modulation is a freely reversible event.

In addition to the *vags*, there exists another set of genes which are regulated reciprocally to the *vags* by the *bvg* locus (27). These *vir*-repressed genes (*vrg-6*, *vrg-18*, *vrg-24*, *vrg-53*, and *vrg-73*) are expressed maximally in avirulent phase-III or C-mode cells (10, 27). In four of the *vrgs*, the repression by bvg appears to be the result of the binding of a 34-kDa *vir*-activated repressor to an intragenic sequence in the *vrg* coding DNA which decreases transcription (10, 11). The function of the proteins encoded by the *vrgs* has not been elucidated; however, a strain mutated in *vrg*-6 was shown to be significantly less virulent in a mouse model of infection (12).

In the veterinary pathogen *B. bronchiseptica*, *bvg* has been shown to negatively regulate the production of flagella (2) and a siderophore (1) resulting in higher expression of these molecules in the avirulent phase. In addition, experiments using phase-locked mutants have shown the virulent phase alone is sufficient for disease production in a rabbit model of infection (15). Similar experiments using phase-locked mutants in a rat model of infection support these conclusions, and furthermore demonstrate that ectopic expression of a normally *vir*-repressed gene product, flagella, impedes bacterial colonization of the trachea (3). Moreover, the investigators found no immunological evidence for modulation to the avirulent phase during the course of infection (3, 15).

However, *B. pertussis* differs greatly from *B. bronchiseptica* being a more fastidious, non-motile, obligate human pathogen which appears not to have an environmental reservoir (49). In addition, a modulating agent which works at concentrations relevant to those which would be found within the human host has not been found (31). Hence, the function of antigenic modulation and the role of the *vrgs* in the life of *B. pertussis* remains a mystery.

We are interested in studying *vir*-repressed surface molecules by first discovering them at the protein level. Surface-exposed *vrg*-products are likely candidates for functional molecules which would interact with host tissues or the environment, and their characterization may help decipher the role (if any) of antigenic modulation *in vivo*. To aid in this work, we have developed a set of B-cell hybridomas which produce MAbs with specificity towards avirulent *B. pertussis* surface proteins. Previous protein work on the phases and modes of *B. pertussis* have focused on the *vir*-activated gene products (7, 17, 48) and only a few potential *vir*-repressed gene products have been shown (10, 37). In this chapter, we describe the identification of *vir*-repressed gene products, including the preliminary characterization of two surface-exposed proteins.

B. MATERIALS AND METHODS

1. Bacterial Strains and Control Antibodies (Abs).

Bordetella strains used in this study are presented in Table II.1. Strains were grown at 37°C on Bordet-Gengou agar (BGA) (Difco Laboratories, Detroit,

MI) containing 13% defibrinated sheep's blood (Triage, Ardrossan, AB, Canada) in 98% humidity. To induce antigenic modulation, strains were grown on BGA containing 5 mM nicotinic acid-20 mM $MgSO_4$ (10). *B. pertussis, B. parapertussis,* and *B. bronchiseptica* were grown for 3 days , 2 days, and 1 day, respectively, before use.

BL-1, anti-LOS A MAb mouse ascites (4), was kindly provided by Dr. Bernard Brodeur, Laboratory Centre for Disease Control, Ottawa, Canada. BPE3, anti-pertactin MAb mouse ascites (14), was kindly provided by Dr. Drusilla Burns, Division of Bacterial Products, Office of Biologics Research and Review, Center for Drugs and Biologics, Food and Drug Administration, Bethesda, MD. A7D12, anti-*B. parapertussis* specific MAb mouse ascites, was developed by this lab (unpublished results). Anti-bacterial alkaline phosphatase antibody was purchased from 5 Prime to 3 Prime, Inc., Boulder, CO.

2. Crude Outer-Membrane vesicles (OMVs) and Triton X-114 Phase Partitioning.

Membranes were prepared by an adaptation of the method of Barenkamp (8). BGA grown *B. pertussis* strains were suspended in Stainer-Scholte broth (SSB) (42) to an $A_{540\ nm}$ of 1.0 in screw-capped tubes (13 by 100 mm) and diluted 1 in 25 in SSB. Broth cultures were grown at 37°C with shaking at 150 rpm for 3 days, and then pelleted by centrifugation at 12 000 x *g* at 4°C. Bacterial pellets were washed with 10 mM *N*-[hydroxyethyl]piperazine-*N*'-[2-

ethanesulfonic acid] (HEPES buffer), pH 8.0, and resuspended in 200 mM lithium chloride-100 mM lithium acetate buffer-0.1 mM phenylmethylsulfonyl fluoride (PMSF), pH 6.0, to a concentration of 0.5 g (wet wt)/ml bacteria. 2 ml suspensions were sonicated in 25 ml Corex tubes in ice water for 8 min using a Branson B-220 ultrasonic cleaner (Branson Cleaning Equipment Co., Shelton, CT). The cells were again pelleted, and the supernatants ultracentrifuged at 125 000 x *g* at 4°C to collect OMVs. OMVs were washed in HEPES buffer by ultracentrifugation at 4°C, and resuspended in HEPES buffer-0.1 mM PMSF. Protein concentrations were determined using the bichloronicotinic acid protein assay (BCA, Pierce Chemical Co., Rockford, IL).

Partitioning of OMVs by Triton X-114 was performed by an adaptation of the method of Forsyth (21). Triton X-114 at 4°C was added to OMVs to 1% final concentration and incubated on ice for 30 min with occasional vortexing for solubilization. The suspension was incubated at 37°C for 30 min to allow detergent condensation and the detergent and aqueous phases separated by centrifugation in a microcentrifuge for 5 min (approximately 10 000 x *g*) at room temperature (RT). The detergent and aqueous phases were extracted as before using HEPES buffer and Triton X-114, respectively. The phases were precipitated by adding 9 volumes of methanol and incubating for 18 h at -70°C followed by centrifugation for 20 min at 4°C in a microcentrifuge. Precipitants were resuspended in HEPES buffer to one half the starting volume of the extracted OMVs.

3. ^{125}Iodination of Whole Organisms.

Radiolabeling was done essentially by the method of Peppler (37). Briefly, organisms were suspended as before to an $A_{540\ nm}$ of 0.15 in 50 mM Tris-43 mM sodium glutamate-90 mM sodium chloride, pH 7.5 (TGS) and 1.5 ml samples centrifuged in a microcentrifuge for 5 min. Pellets were resuspended in 50 µl TGS and added to glass culture tubes previously coated with 1 µg Iodogen catalyst (Pierce). A 1.5 µCi/µl ^{125}I solution was prepared from Na^{125}I (100 µCi/µl, Amersham Life Sciences, Oakville, ON, Canada) diluted in TGS and 10 µl added to the culture tubes. The labeling reaction proceeded for 10 min with occasional swirling at RT. Labeled bacteria were washed 3 times with 1 ml TGS by centrifugation in a microcentrifuge to remove unincorporated ^{125}I. ^{125}I-labeled proteins were detected in dried SDS-PAGE gels by autoradiography using Kodak X-Omat-R film (Eastman Kodak Co., Rochester, NY) with a Dupont Cronex Lightning Plus intensifying screen (E.I. duPont de Nemours & Co., Wilmington, DW) and exposure at -70°C.

4. SDS-PAGE.

SDS-PAGE was performed by the method of Laemmli (29) using the modifications of Peppler (37). Whole cell lysates were prepared by suspending bacteria as before to an $A_{540\ nm}$ of 0.15 in TGS and 1.5 ml samples centrifuged for 5 min in a microcentrifuge. 50 µl of Laemmli's solubilization buffer was added to each pellet followed by brief vortexing and sonication, and incubation in a

boiling waterbath for 5 min. Pelleted ^{125}I labeled bacteria (as described above) were processed in the same manner. To destroy protein epitopes before electrophoresis, whole cell lysates were digested with 10 μg proteinase K (Boehringer Mannheim Canada, Laval, PQ, Canada) at 56°C for 30 min with vortexing at 5 min intervals. 5 μl and 10 μl samples were loaded per lane of mini-gels (5.5 cm by 8 cm) and large slab gels (12 cm by 14 cm), respectively. OMVs were solubilized for SDS-PAGE by addition of 1/5 volume of 5-times strength Laemmli's solubilization buffer followed by incubation in a boiling waterbath for 5 min.

An acrylamide stock with a 30:0.8 ratio of acrylamide to *N,N,'*-methylenebisacrylamide was used in the preparation of acrylamide gels which had 4% total acrylamide for the stacking gel, and between 7.5% to 16% total acrylamide for the separating gels depending on the separation desired. For large slab gels (Hoeffer SE 600 apparatus, Hoeffer Scientific Instruments, San Francisco, CA), 1.5 mm spacers were used and electrophoresis conducted for approximately 4 h at 10 W per gel constant power with cooling to 4°C. For mini-gels (Mini-Protean-II Cell, Bio-Rad, Hercules, CA), 0.75 mm spacers were used and electrophoresis conducted for approximately 1 h 10 min at 5 W constant power without cooling. Apparent molecular weights (MW) were determined by comparison with either low molecular weight protein standards (Bio-Rad), or pre-stained low molecular weight protein standards (Gibco-BRL, Burlington, ON, Canada). To visualize proteins, gels were stained with Coomassie Brilliant Blue

R-250 (Bio-Rad) as described (37).

5. Two-Dimensional Gel Electrophoresis (2DGE).

2DGE was performed essentially by the method of O'Farrell (36). OMV solutions were suspended 5:1 in 7.5 M urea-2.0% Triton X-100-5% β-mercaptoethanol-1.6% 5/7 Bio-Lyte ampholyte (Bio-Rad)-0.4% 3/10 Bio-Lyte ampholyte (Bio-Rad) (first-dimension sample buffer), vortexed, and incubated for 20 min at RT. An equivalent of 100 μg protein of OMV was loaded for each isoelectric focusing (IEF) tube. Pelleted ^{125}I labeled bacteria (as described above) were suspended in 50 μl first-dimension sample buffer, solubilized with repeated tituration and sonication at RT for 20 min, and entire samples loaded individually onto IEF gels. IEF was performed in a Protean-II tube gel apparatus (BioRad) using 100 mm long by 1.0 mm internal diameter capillary gels. IEF gels consisted of 9.2 M urea-4% acrylamide-20% Triton X-100-1.6% 5/7 Bio-Lyte ampholyte-0.4% 3/10 Bio-Lyte ampholyte. The catholyte and anolyte were 0.1 M NaOH and 0.06% phosphoric acid, respectively. IEF was conducted for 16 h at 400 V followed by 2 h at 800 V. The pH range of extruded IEF gels was estimated by cutting blank gels into approximately 5 mm segments, and measuring the pH of each segment using a pH meter after vortexing and equilibration for 30 min at RT in 2 ml degassed distilled H_2O. Extruded IEF gels were equilibrated in Laemmli's solubilization buffer at RT for 20 min and then electrophoresed in the second-dimension by SDS-PAGE as described above

using 1.5 mm thick slab gels whose separating gels were 15% acrylamide.

6. Western Immunoblotting.

Western blotting to nitrocellulose (Bio-Rad) was performed by an adaptation of the method of Batteiger (9) in 25 mM sodium phosphate buffer, pH 7.4, for 18 h at 27 V at 4°C using a submarine apparatus (Trans-Blot Cell, Bio-Rad). Blots were blocked in 25 mM Tris-buffered-saline (TBS)-1% skim milk-3% bovine serum albumin (BSA)-0.05% Tween-20, pH 7.2 before probing with Abs. Where required, Ab solutions were diluted in TBS-3% BSA. After incubation with primary Abs (1° Abs), blots were washed 6 times for 10 min each in 0.85% NaCl, and then incubated with secondary Ab (2° Ab). 2° Ab for westerns and ELISAs was low cross-reactivity, goat anti-mouse IgG (whole molecule)-alkaline phosphatase conjugate (Catalogue no. #115-055-062, Jackson Immunolaboratories, Mississauga, ON, Canada). Blots were washed in 0.85% NaCl and developed using the Immun-Blot alkaline phosphatase assay kit (Bio-Rad).

7. OMV-ELISA and Whole Cell ELISA (WC-ELISA).

To coat antigen (Ag) for OMV-ELISA, OMV solutions were diluted to 5 μg protein/ml in 10 mM phosphate buffered saline, pH 7.4 (PBS) and 100 μl per well incubated in ELISA plates for 18 h at 4°C. For WC-ELISA, bacteria were suspended from BGA plates as before to an $A_{540\ nm}$ of 0.15 in PBS-0.1%

Thimerosol and killed at 56°C for 30 min. Suspensions were distributed 100 µl per well and incubated for 18 h at 4°C to coat ELISA plates. After coating, plates were washed three times with 0.85% NaCl-0.05% Tween-20 to remove unbound Ag. Where required, Ab solutions were diluted in PBS-0.5% BSA-0.05% Tween-20-2% heat-inactivated fetal bovine serum (FBS; Flow Laboratories, McLean, VA). 1° Abs were incubated for 2 h at 4°C followed by three washes with 0.85% NaCl-0.05% Tween-20. Plates were incubated with alkaline phosphatase conjugated 2° Ab for 1 h at 37°C followed by washing with 0.85% NaCl-0.05% Tween-20. Plates were developed using the Sigma 104 phosphatase system (Sigma Chemical Co., St. Louis, MO). BL-1 ascites, BPE3 ascites, A7D12 ascites, and SP2/0 (ATCC CRL 1581) culture supernatant were used as controls by which the specificity of test Ab solutions were based.

8. Generation of Hybridomas.

B. pertussis strain BP347 (phase-III-like) was suspended as before in 0.85% NaCl-0.01% Thimerosol-0.01% erythromycin to an $A_{540\ nm}$ of 0.12 (approximately 1 x 10^9 cells/ml) and incubated for 3 h at 37 °C. Killed cells were washed twice in 0.85% NaCl by centrifugation in a microcentrifuge and resuspended in PBS. 4 to 6 week-old female Balb/c mice were injected intraperitoneally (i.p.) with approximately 3 x 10^9 cells each. Mice were boosted intravenously (i.v.) with approximately 3 x 10^7 cells 3 weeks after primary immunization. Serum Ab titers by WC-ELISA were determined to be greater than

1/1000 at 10 days post-boost. The fusion mouse was boosted 3 days before splenectomy and fusion. SP2/0 cells were fused with mouse splenocytes to generate hybridomas by standard hybridoma technology (25).

In a second fusion protocol, mice were immunized with BP347 protein eluted from SDS-PAGE. The protein bands eluted were between 13- to 19-kDa. This immunogen was chosen based on the reactivity of a MAb (14EG12E3) generated in the first fusion, and identified unique bands in avirulent *B. pertussis* OMV protein profiles by SDS-PAGE. Protein bands were electroeluted using Laemmli's electrophoresis buffer (29) at 5 W constant power for 4 h at 4°C using the Little Blue Tank apparatus (Isco, Inc., Lincoln, NE) with 2 000 dalton cutoff cellulose dialysis tubing (Spectrum Medical Industries, Los Angeles, CA). Proteins were precipitated by addition of 5 volumes 80% acetone, followed by incubation in a dry-ice bath for 5 min and centrifugation for 2 min at 4°C in a microcentrifuge. The precipitant was resuspended in 1 ml 80% acetone, reprecipitated, and resuspended in PBS. Protein concentrations were determined as described above. The presence of the Ag of interest was confirmed by Western immunoblotting with 14EG12E3 MAb. Mice were immunized 3 times i.p. with approximately 5 μg eluted protein and 5 μg Quil-A Saponin (Cedarlane Laboratories Hornby, ON, Canada) at three week intervals until serum Ab titers were greater than 1/1000 by OMV-ELISA. 3 days prior to splenectomy, 3 fusion mice were each boosted i.v. with 50 μg BP347 OMV diluted in PBS. Hybridomas were generated as before using 3 spleens with a 3-

fold increase of all reagents. Hybridomas were selected, maintained, single cell cloned by limiting dilution, and stored by standard techniques (25). Mouse ascites was produced by standard techniques (25).

Hybridomas of interest were chosen by screening for specificity of their supernatants to react with BP347 (phase-III-like) versus *B. pertussis* strain BP338 (phase-I) in WC-ELISA (fusion 1) and OMV-ELISA (fusion 2). Hybridomas were isotyped with the ImmunoSelect isotyping kit (Gibco-BRL, Canada). The Ags recognized by the MAbs were classified by Western immunoblotting, WC-ELISA, and OMV-ELISA.

9. Immunoelectron Microscopy.

Bacteria from BGA were washed 3 times in PBS and adsorbed to formvar coated 200 mesh copper electron microscope grids. Adsorbed bacteria were blocked in PBS-1% BSA, and then incubated with MAbs diluted in PBS-1% BSA. Samples were blocked again, and then incubated with a secondary goat anti-mouse IgM-IgG mixture conjugated with 10 nm gold particles (EY Labs, San Mateo, CA). Samples were washed twice in PBS, rinsed in distilled H_2O and edge blotted with Whatman filter paper (Whatman, Maidstone, England). Samples were examined unstained and photographed using a Philips model 410 transmission electron microscope.

C. RESULTS

1. Avirulent *B. pertussis* specific proteins.

We used SDS-PAGE analysis of *Bordetella pertussis* isogenic strains BP338 and BP347 to identify protein bands increased in the avirulent phenotype (BP347) relative to the virulent phenotype (BP338). As seen in Fig. II.1 no avirulent specific bands are apparent in whole cell lysates (lanes 1 and 2). However, SDS-PAGE protein profile analysis of OMVs using 15% separating gels reveals at least eight bands which are at a lower concentration or absent in BP338 relative to BP347 (Fig. II.1, lanes 3a to 4b). Most of these proteins are of low molecular weights (less than 30-kDa). SDS-PAGE analysis using 7.5% separating gels did not reveal any avirulent specific OMV bands in the higher molecular weight range (Appendix, Fig. A.1).

Further resolution of OMV proteins was obtained by separation on the basis of isoelectric points and molecular weight using 2DGE of BP338 and BP347 OMVs. This method resolved at least twenty-two proteins which are absent or at lower concentration in BP338 relative to BP347 (Fig. II.2A and Fig. II.2B). As seen with one-dimensional SDS-PAGE, most of these proteins are of low molecular weight; however a number of proteins with apparent molecular weights of 30-kDa or more are seen by 2DGE. 2DGE using low-percentage SDS-PAGE separating gels to resolve higher molecular weight proteins was not performed. The pH range of the IEF gels was routinely between pH 5 and 7 and the distribution of the avirulent specific spots was not clustered in any single

portion of the pH gradient.

2. Avirulent *B. pertussis* specific MAbs.

We developed MAbs with specificity for avirulent *B. pertussis* surface Ags to use as molecular tools to monitor antigenic modulation of *B. pertussis* and to characterize *vir*-repressed surface proteins. We initially used whole cells as an immunogen in the production of hybridomas so that all potential Ags might be included. We screened hybridoma supernatants against whole cells of virulent strain BP338 and avirulent strain BP347 by ELISA to identify MAbs with specificity for the surface of the avirulent cells. The three fusions done in this manner only yielded one hybridoma 14EG12E3, whose MAb recognizes a band with an apparent molecular weight of 14.5-kDa by Western immunoblotting (Appendix, Fig. A.2). Unfortunately, this hybridoma clone is unstable and becomes unproductive on repeated passage.

In a subsequent fusion we used enriched BP347 protein containing the 14.5-kDa Ag (*vir*-repressed Ag-a or vra-a) as an immunogen to generate hybridomas. We screened over one thousand uncloned hybridoma culture supernatants against BP347 and BP338 OMVs by ELISA to identify MAbs with specificity for avirulent cells. After subcloning, this fusion yielded thirteen hybridomas whose Abs have specificity for avirulent *B. pertussis*. Isotyping showed IgM to be the predominant class of the cloned hybridomas with only two being of the IgG class (Table II.2).

The phenotype specificity of the Abs was confirmed by testing their ability

to react with avirulent BP347 and virulent BP338 by both WC-ELISA and OMV-ELISA. Twelve of the hybidoma supernatants produced a positive reaction with BP347 OMVs and whole cells, and a negative reaction against BP338 OMVs and whole cells. Two supernatants, 2B12 and 6G4, although they reacted with BP338, produced ELISA values against BP347 that were at least twice the ELISA values against BP338 by both OMV-ELISA and WC-ELISA. In addition, supernatants from hybridomas 1G7, 2C3A13, 4F10, 9H1, 13A8, 20B12, and 7H1A1 were tested by WC-ELISA against BP338 and modulated BP338 and react specifically with modulated *B. pertussis*.

All culture supernatants were tested for their ability to react against BP347 and BP338 whole cell lysates after Western blotting to nitrocellulose. Supernatants from 2B12, 6G4, and 20D9 do not react by Western immunoblotting (data not shown). 7H1A1 supernatant reacts weakly with a 17-kDa band in BP338 while reacting strongly with the same band in BP347 (Fig. II.3C, lanes 1 and 2). We have called this Ag *vir*-repressed-Ag-b (vra-b). 7H1A1 also reacts weakly with the 14.5-kDa vra-a in BP347 (Fig. II.3C, lanes 1 and 2). The other eight Abs react specifically with the 14.5-kDa Ag (vra-a) in BP347 but do not react with BP338 (for example MAb B13A8, Fig. II.3B, lanes 1 and 2). In addition, we probed Western blots of unmodulated and modulated *B. pertussis* strains 18323, UAH 14797, and UAH 9775 with MAb B13A8 and found the 14.5-kDa vra-a to be expressed only in the modulated bacteria (Appendix, Fig. A.3 and Fig. A.4).

To test the specificity of the Abs for *B. pertussis* we probed Western blots

of virulent and avirulent isogenic pairs of *B. parapertussis* and *B. bronchiseptica*. MAb B13A8 does not recognize any molecules of *B. parapertussis* or *B. bronchiseptica* by this method (Fig. II.3B lanes 3 to 6). MAb 7H1A1 recognizes a 17-kDa vra-b-like band in these species but with a lower intensity than seen in avirulent *B. pertussis* which is not *vir*-regulated (Fig. II.3C lanes 3 to 6). To further confirm the species specificity of the Abs, we tested supernatants from hybridomas 1G7, 2C3A13, 4F10, 9H1, 13A8, 20B12, and A17H1 by WC-ELISA against *B. parapertussis* and *B. bronchiseptica* strains grown under modulating and non-modulating conditions. The bacteria tested were *B. parapertussis* strains 17903, 77, and 504, and *B. bronchiseptica* strains Rat 1, 501, and Columbus. The only hybridoma supernatant which reacts by this method against *Bordetellae* species other than *B. pertussis* is 7H1A1. This MAb shows reactivity against phase-III strain Columbus. A summary of these data is presented in Table II.2.

3. Characterization of *vir*-repressed Ags.

We tested *B. pertussis* strain 18323 and its Tn*PhoA*::*vrg* derivatives SK6, SK18, SK24, SK53, and SK73 to determine if the vras identified in this study are the protein products of the *vrgs* described previously (10-12, 27). Western immunoblots of these strains grown under modulating and non-modulating conditions were done with MAbs B13A8 and 7H1A1, and polyclonal anti-bacterial alkaline phosphatase antiserum. The vras are regulated in the mutant strains as they are in the 18323 parent and appear at their usual apparent molecular

weights (Appendix, Fig. A.4). The results with anti-bacterial phosphatase were the same as described previously (Fig. 1 of reference 12) and did not reveal any *PhoA* fusion products whose apparent molecular weight would approximate the combined molecular weights of a vra and an alkaline phosphatase insert (data not shown).

Confirmation of the proteinaceous nature of the vras was performed by Western immunoblotting proteinase K treated samples of modulated *B. pertussis* strains UAH 14797 and UAH 9775. Proteinase K digestion eliminates reactivity with MAbs B13A8 (anti-vra-a) and 7H1A1 (anti-vra-b) (Appendix, Fig. A.5).

To further characterize vra-a and vra-b we used Western immunoblotting of BP347 OMVs after separation by 2DGE. As seen in Fig. II.4A, vra-a is an acidic 14.5-kDa protein. This spot corresponds to a Coomassie stained spot by 2DGE ("a" in Fig. II.2B). In addition to the 14.5-kDa spot two larger spots at the same isoelectric points are seen by this method (Fig. II.4A). A tailing effect is also seen from the vra-a band across the entire pH range starting from its acidic location. Fig. II.4B demonstrates that vra-b is a relatively basic 17-kDa protein. There is no corresponding Coomassie staining spot by 2DGE for vra-b (Fig. II.2B). The MAb 7H1A1 used to demonstrate vra-b also cross reacts with vra-a, and thus it also labels the acidic vra-a spots.

We further distinguished vra-a from vra-b by testing their ability to extract into the hydrophobic detergent Triton X-114. Western Immunoblotting demonstrates that vra-a does not extract into the detergent phase whereas at

least half of vra-b does (Fig. II.5).

4. Surface-exposure of *vir*-repressed Ags.

We confirmed vra-a and vra-b as surface molecules using immunoelectron microscopy. Fig. II.6 demonstrates that both vras are found on the surface of avirulent *B. pertussis* but not virulent *B. pertussis*. Control MAbs demonstrate LOS to be on the surface of both virulent and avirulent cells, and pertactin to be on the surface of only virulent cells. In addition, a fluorescence immunolabeling technique (22) was used to confirm the results of the immunoelectron microscopy (data not shown).

^{125}Iodine-labeling was used to further demonstrate surface-exposed molecules of virulent and avirulent *B. pertussis*. Autoradiograms of SDS-PAGE profiles demonstrate four bands with increased surface labeling in the avirulent phenotype (Fig. II.7). These bands are of similar molecular weights to the vras. These profiles agree closely to phase-I and phase-III *B. pertussis* surface labeled profiles seen previously (Fig. 5B of reference 37). Using the higher resolving power of 2DGE it was possible to demonstrate an intensely labeled spot which corresponds to vra-a in avirulent *B. pertussis* (Fig. II.8B). This same spot is seen only faintly in the virulent phenotype (Fig. II.8A). A labeled spot which would correspond to vra-b is not seen by this method. Using Increased exposure times it is possible to demonstrate ^{125}Iodine-labeling of other surface molecules which are increased in avirulent *B. pertussis* (data not shown).

E. DISCUSSION

The ability to coordinately regulate gene expression is common to a number of bacterial pathogens (5, 33). Coordinate regulation allows a pathogen to express genes specifically when the gene products are appropriate for an environmental niche, thus saving metabolic energy in contrast to constitutive production. In addition, the global nature of such regulation allows differential expression of sets of genes at crucial times during the organism's life. These switches in gene expression may be important in such processes as transmission from host to host, survival in the environment, colonization, pathogenesis, evasion of the host immune system, and persistence in host microenvironments. Our goal is to further characterize *B. pertussis* molecules that are maximally expressed in the C-mode phenotype as the result of coordinate regulation, with the ultimate goal of understanding the relevance and function of these molecules and their regulation.

In *Bordetella pertussis* there are a number of virulence factors which contribute to disease production in humans (49). Not surprisingly, a number of these factors including pertussis toxin, adenylate cyclase toxin, filamentous hemagglutinin, and pertactin are coordinately regulated by the *bvg* (*Bordetella* virulence gene) locus and are thus subject to modulation (24, 41, 44, 45, 50). Many of these *vir*-activated gene products have well defined functions, and some are required for virulence in a variety of models (49). Hence, the ability to regulate the production of these factors in response to environments in the host

would be appropriate for the bacterium in terms of temporal need and metabolic efficiency.

The functions and purpose of the *vir*-repressed gene products of *B. pertussis* are poorly understood compared to those of the *vir*-activated gene products. Significant homology with known molecules at the DNA or predicted protein level has not been found for any of the characterized *vrgs* (11). However, Beattie *et al.* (11) have shown at least one gene, *vrg-6*, to be required for full virulence in a mouse model of infection. The investigators propose that the *vrg-6* product may act as an accessory colonization factor based on the predicted protein's sequence homology to known protein structural motifs, and the reduced ability of a *vrg-6* mutant to colonize lungs and trachea in the mouse model.

Moreover, the investigators found that although sequences similar to *vrg-6* appeared in *B. parapertussis* and *B. bronchiseptica*, the gene was expressed only in *B. pertussis* . This may lead one to speculate that *vrg-6* plays a specific role in *B. pertussis*' life. However, these data should be viewed with caution because the strains used in these studies are derivatives of strain 18323 which has been noted as being atypical of the species (35). Since *vrg-6* is negatively regulated by *bvg*, its protein product would only act in an environment which induced the BvgAS system to reduce the expression of the *vags*, and derepress the expression of the *vrgs*. When and where this might occur in the life of *B. pertussis* is unknown.

We have been able to identify a number of molecules which are increased in the avirulent (C-mode-like) phenotype relative to the virulent (X-mode)

phenotype: eight by SDS-PAGE of OMVs, and twenty-two by 2DGE of OMVs. To our knowledge, this is the first demonstration of such a large number of *vir*-repressed molecules in *B. pertussis*. Differences in our methodology likely account for our results. We enriched for outer membrane proteins by differential centrifugation without detergent extraction. Although this likely means our preparations contain inner membrane contaminants, artifactual loss of outer membrane components by detergent extraction would not have occurred. It has been noted that sucrose gradient isolation of *B. pertussis* outer membranes is not reliable based on the monitoring of defined enzymatic membrane markers (17). We believe that detergent methods of outer membrane purification should also be used with caution in *B. pertussis* until definitive evidence of their reliability is proven.

Beattie *et al.* have demonstrated up to eight proteins which are increased in C-mode cells using a enzymatic, freeze-thaw method of membrane isolation (10). Our protein profiles differ considerably from theirs, and this is likely due to the different method of membrane extraction used. However, several notable similarities are evident in the lower molecular weight range (less than 30-kDa), and many of the bands seen by both methodologies may be identical. It is plausible that differences in SDS-PAGE techniques between investigators has resulted in the differences in the amount of *vir*-repressed proteins identified in the literature. Moreover, the higher resolving power of 2DGE has allowed us to demonstrate many new *vir*-repressed proteins. It is our intention to further characterize the ability of different methods to identify *vir*-repressed proteins, and

determine their ability to segregate different components of the *B. pertussis* membrane.

The ability of *Bordetella pertussis* to reversibly alter its phenotype in response to environmental conditions was first demonstrated immunologically (28). Lacey detected modulation using cross-adsorbed antisera developed in animals against *B. pertussis* grown under different culture conditions. However, the C-mode was noted to be poorly immunogenic. Indeed, Lacey found that detectable antibody titers against C-mode organisms are only generated by immunization of avian species and not mammalian species. Lacey also noted that two human convalescent sera from adults with atypical pertussis agglutinated C-mode cells but not X-mode cells. However, the methods used would not have been able to differentiate between a specific agglutination reaction against unique C-mode Ags and a specific agglutination reaction with C-mode cells via Ags common to both the C- and X-modes. Although evidence for a convalescent Ab response to avirulent or C-mode Ags in humans is lacking, it is possible that the techniques used in this study to detected *vir*-repressed proteins may be adapted to detect such a response.

Since Lacey's work, other attempts to raise antisera against *B. pertussis* C-mode Ags have not been successful. Beattie *et al.* proposed that the poor predicted antigenic index of the *vrg-6* product likely explains the inability to raise antisera against it (11). It has been hypothesized that a switch to the poorly antigenic C-mode state through modulation might be a mechanism by which *B. pertussis* can avoid immune surveillance. Another explanation for the

absence of significant anti-C mode Abs in convalescent sera is that switch to C-mode may occur in a site within the human host which protects the organism from recognition by the immune system.

Similarly, we have found it difficult to generate C-mode specific murine MAbs, and several fusions were required to develop a hybridoma bank specific for vras. The Abs produced recognize all *B. pertussis* strains tested, including two clinical isolates which were of the most common epidemiological types seen in a recent whooping cough outbreak (16). The most common protein recognized by our Abs is the 14.5-kDa vra-a, which is the Ag that was enriched for in the second immunization protocol. This Ag was recognized by ten of fourteen Abs we generated and is shown to be surface-exposed by ^{125}Iodine surface labeled 2DGE profiles, and by immunolabeling of whole cells by three separate techniques. The predominant isotype class of the anti-vra-a Abs is IgM, which could lead one to speculate that the Abs recognize a T-cell independent epitope. However, it should be noted that the first MAb raised against this molecule was an IgG molecule. Until competition assays are performed with purified MAbs it will not be clear if the different anti-vra Abs we developed recognize common or separate epitopes on vra-a.

Further characterization has shown vra-a to be an acidic protein by Western immunoblotting after 2DGE. Moreover, the specific appearance of comigrating protein in avirulent Coomassie stained 2DGE profiles suggests that anti-vra-a Abs recognize a *vir*-repressed protein and not a *vir*-repressed post-

translational modification of a constitutively expressed protein. However, it is possible that a *vir*-repressed modification could alter the mobility of a preexisting protein, and thus make it appear as a unique band in protein profiles.

The presence of an acidic molecule of approximately twice the molecular weight of vra-a is apparent by 2DGE in Western immunoblots and Coomassie stained gels (Fig. II.2B and Fig. II.4A). This band is possibly a dimer of the vra-a molecule which is not dissociated because high amounts of protein coupled with solubilization at ambient temperatures overwhelms the denaturing and reducing capacity of the 2DGE system. Alternatively, the higher molecular weight protein could be distinct from the 14.5-kDa protein, and is only detected with the increased amounts of protein we separated by 2DGE. A tailing effect is also seen extending from vra-a when it is detected by 2DGE immunoblotting of OMVs. This is likely unfocused Ag which results from the high amount of protein loaded onto first dimension IEF gels. The doublet appearance of vra-a at around 14.5-kDa in immunoblots of 2DGE is occasionally seen by one-dimensional SDS-PAGE analysis of avirulent OMVs and whole cells (Appendix, Fig. A2) and is as yet unexplained.

The other vra which is detectable by immunoblotting is vra-b. Our results indicate this is a distinct molecule from vra-a based on differences in pI, molecular weight, and Triton X-114 solubility. However, the ability of the anti-vra-b MAb (7H1A1) to cross-react with vra-a may suggest some relatedness of the Ags. Like vra-a, vra-b is surface-exposed based on immunological data. However, this protein is refractile to detection by Coomassie staining and

^{125}Iodine surface labeling. Hence, we have not been able to determine if the *vir*-specificity of the epitope is at the translational or conformational levels. In addition to vra-a there are several other *vir*-repressed molecules which are surface-exposed based on ^{125}Iodine-labeling.

The vras appear not to be products of the characterized *vrgs* because they are neither destroyed nor have increased molecular weights in mutants strains which contain *vrg*::Tn*PhoA* fusions. It is notable that vra-a is tightly regulated (at least at the translational level). This is in contrast to the less strict regulation seen with the four *vrgs* (including *vrg-6*) which contain repressor binding sites (10, 27). It is interesting to speculate that the gene encoding vra-a may be regulated by the same mechanism as *vrg-73*, which is tightly regulated and does not contain the sequence for repressor binding. Genetic testing of this hypothesis will require cloning of the gene which encodes vra-a. The predicted molecular weight of the *vrg-6* protein (approximately 11-kDa, reference 11) is close to the apparent molecular weight of vra-a (14.5-kDa). Thus, cloning of the vra genes will be needed to definitively prove the vras are not products of the characterized *vrgs*.

The regulation of vra-b between the avirulent and virulent phenotypes of *B. pertussis* is less strict than vra-a with detectable levels of this Ag observed in virulent *B. pertussis*. This is similar to the regulation of the *vrgs* which contain the repressor binding site (10). Unlike the *vrg-6* transcript (11) and the vra-a protein, vra-b is detectable in other *Bordetellae* species. However, the amount of

Ag is less in *B. bronchiseptica* and *B. parapertussis* than in *B. pertussis* and is not *vir*-regulated. These observations are based on immunological data and thus it is possible that proteins that are homologous to the vras exist in the other *Bordetellae* species tested, but they do not react strongly with the anti-vra Abs. Cloning of the vra structural genes would allow us to test if the Ab reactions reflect the true level of proteins in the different species.

Studies with the veterinary pathogen, *B. bronchiseptica*, have indicated that the virulent (X-mode) phase is sufficient for disease production, and the authors found no immunological evidence for modulation *in vivo* (3,15). However, *B. pertussis* differs in its host range, and its ability to survive in the environment (15, 49). In addition, the *vrg* products appear to be distinct between these two species (44). Hence, it is reasonable to speculate that antigenic modulation may have evolved in response to the different requirements of these species. Efforts are under way to further characterize the *vir*-repressed proteins of *B. pertussis* by biochemical, genetic, and reverse-genetic techniques with the goal of further understanding the regulation and function of these molecules. In addition, the avirulent specific Abs described may serve as valuable tools for searching for modulation *in vitro* and *in vivo*. It is hoped that further investigations into the process and products of antigenic modulation will lead to a better understanding of the survival mechanisms of this intriguing human pathogen.

TABLE II.1. *Bordetella* strains used in this study

Strain	Relavant feature(s)	Source and/or reference
B. pertussis		
BP338	Nal^r derivative of clinical isolate Tohama I	Dr. A. Weiss (50)
BP347	BP338 *bvgS1*::Tn*5*	"
18323	mouse challenge strain	ATCC 9797
SK6	18323 *vrg-6*::*TnphoA*	Dr. J. Mekalanos (27)
SK18	18323 *vrg-18*::*TnphoA*	"
SK24	18323 *vrg-24*::*TnphoA*	"
SK53	18323 *vrg-53*::*TnphoA*	"
SK73	18323 *vrg-73*::*TnphoA*	"
UAH 14797	clinical isolate	Provinicial Laboratory of Northern Alberta, Edmonton, AB, Canada (35)
UAH 9775	clinical isolate	"
B. parapertussis		
P14	clinical isolate	Dr. R. Rapuoli (6)
P15	spontaneous phase-III derivative of P14	Dr. R. Rapuoli (24)
17903	clinical isolate	Dr. J. Munoz, Rocky Mountain Laboratories, Hamilton, MT (35)
77	clinical isolate	"
504	clinical isolate	Dr. H. Ackerman, Dept. of Microbiology, Univ of Laval, Laval, PQ, Canada
B. bronchiseptica		
110 H	host species: dog	Dr. D. Bemis, Dept. of Microbiology, Univ of Tennessee, Knoxville, TN (35, 38)
110 NH	spontaneous phase-III derivative of 110 H	(38)
Rat 1	host species: rat	(35, 38)
501	host species: dog	"
Columbus	host species: cat, phase-III isolate	"

TABLE II.2. Summary of antibodies with specificity for phase-III and modulated *B. pertussis*

Antibody	Isotype	*vir* (+)[a] OMV ELISA	*vir* (-)[b] OMV ELISA	*vir* (+) WC ELISA	*vir* (-) WC ELISA	Blots[c]	Ag mol wt (KDal)	*B. pertussis* specific[d]
1G7	IgM	-	+	-	+	yes	14.5	yes
2C3A13	IgM	-	+	-	+	yes	14.5	yes
4F10	IgM	-	+	-	+	yes	14.5	yes
9H1	Mixed[e]	-	+	-	+	yes	14.5	yes
13A8B	IgM	-	+	-	+	yes	14.5	yes
13H2B1	IgM	-	+	-	+	yes	14.5	yes
14EG12E3	IgG_3	-	+	-	+	yes	14.5	yes
16D9A5	IgM	-	+	-	+	yes	14.5	yes
20B12	IgM	-	+	-	+	yes	14.5	yes
22F5	Mixed	-	+	-	+	yes	14.5	yes
2B12	Mixed	+	2x[f]	+	2x	no	ND[g]	ND
6G4	IgM	+	2x	+	2x	no	ND	ND
20D9	IgM	-	+	-	+	no	ND	ND
7H1A1	IgG_1	-	+	-	+	yes	17	no

a. phase-I unmodulated *Bordetella* species.
b phase-III or modulated *Bordetella* species.
c. Ag detectable by Western immunoblotting.
d. Reacts with *B. pertussis* but not with the *B. bronchiseptica* or *B. parapertussis* strains tested.
e. Uncloned hybridoma which is not isotype monospecific.
f. ELISA values are twice that found with the *vir* (+) strain.
g. Not determined.

FIGURE II.1

Comparison of protein profiles of virulent (phase-I) *B. pertussis*, and avirulent (phase-III-like) *B. pertussis*. I.

Coomassie stained 15% SDS-PAGE. Numbers on the vertical axis represent the apparent molecular weights of protein standards in kDa. MW, Bio-Rad protein standards; 1, virulent phase BP338 whole cell lysate; 2, avirulent phase BP347 whole cell lysate; 3a, 15 μg BP338 OMVs; 3b, 30 μg BP338 OMVs; 4a, 15 μg BP347 OMVs; 4b, 30 μg BP347 OMVs. Arrows indicate positions of bands which are increased in avirulent phase OMVs relative to virulent phase OMVs.

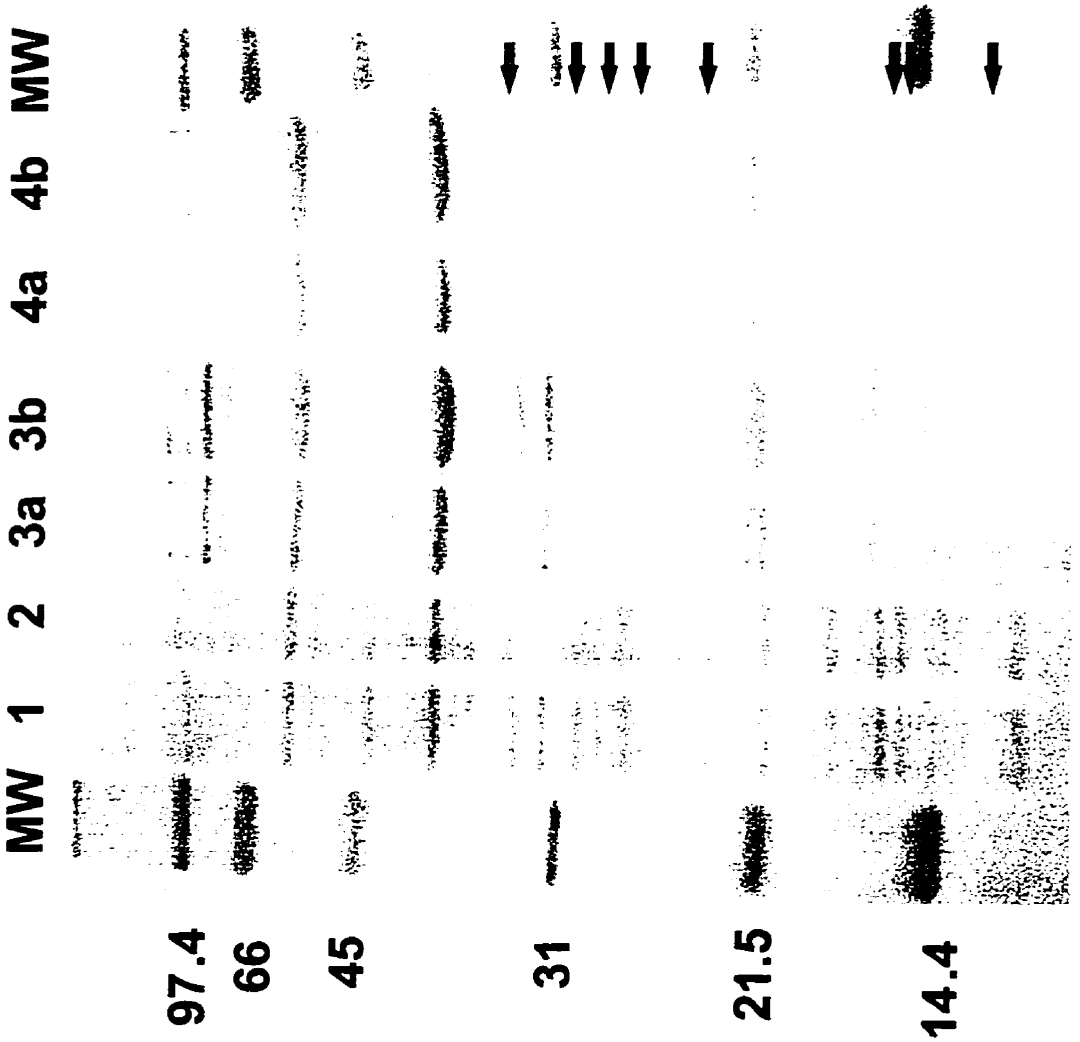

MW
1
2
3a
3b
4a
4b
MW
97.4
66
45
31
21.5
14.4

FIGURE II.2

Comparison of protein profiles of virulent (phase-I) *B. pertussis*, and avirulent (phase-III-like) *B. pertussis*. II.

Coomassie stained 15% SDS-PAGE. Numbers on the vertical axis represent the apparent molecular weights of protein standards in kDa.

A. Coomassie stained 2DGE of strain BP338 OMVs.

B. Coomassie stained 2DGE of strain BP347 OMVs. White or black stars indicate spots which are increased in avirulent phase OMVs relative to virulent phase OMVs.

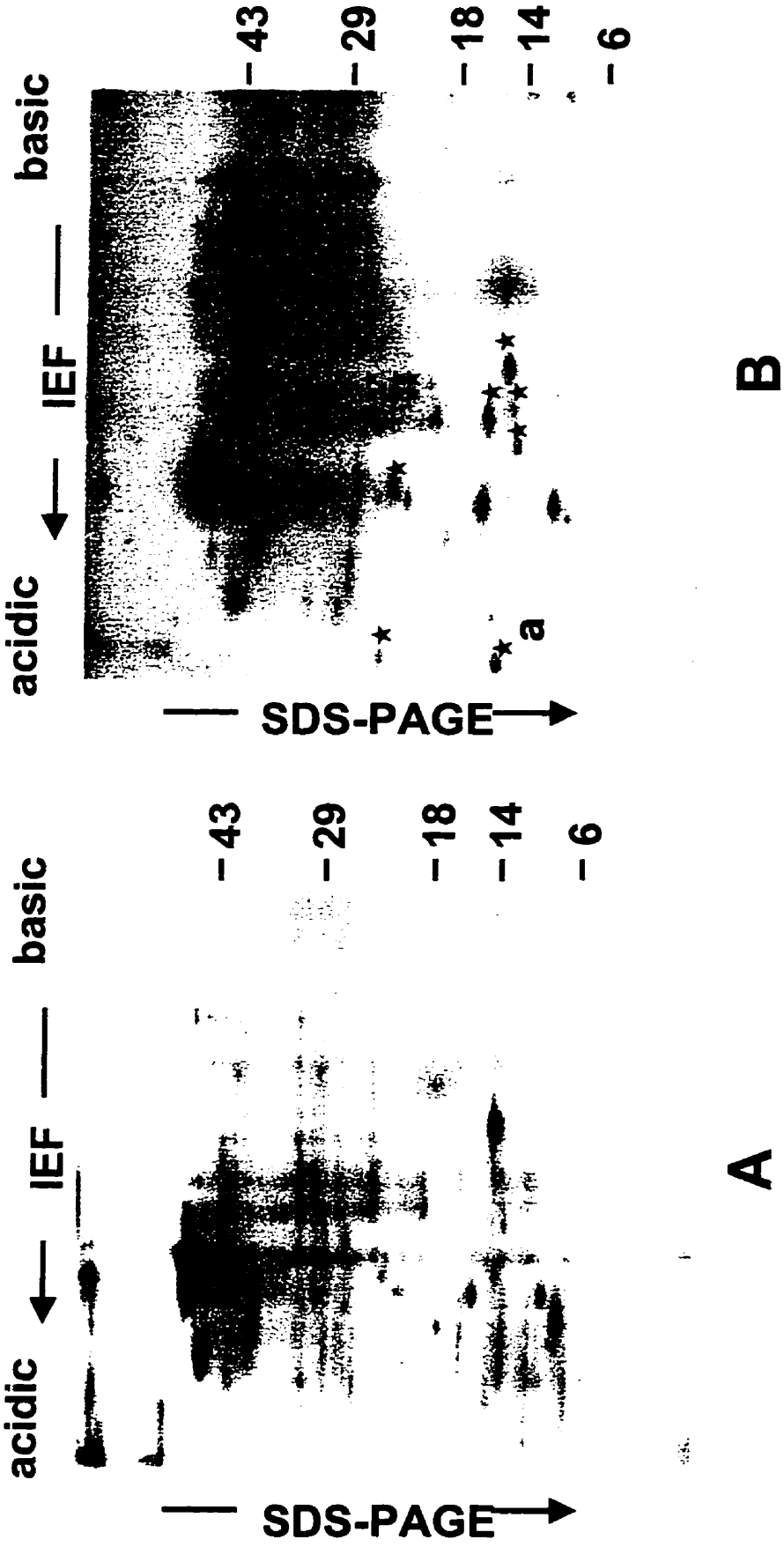

B
acidic
IEF
basic
43
29
18
14
6
a
SDS-PAGE
A
acidic
IEF
basic
43
29
18
14
6
SDS-PAGE

FIGURE II.3

Western immunoblot analysis of species and phase specificity of MAbs 7H1A1 and 13A8B.

A. Coomassie stained 15% SDS-PAGE of whole cell lysates. Numbers to the left of the figure represent the apparent molecular weights of protein standards in kDa. MW, Gibco-BRL protein molecular weight standards; 1, *B. pertussis* BP338; 2, *B. pertussis* BP347; 3, *B. parapertussis* P14; 4, *B. parapertussis* P15; 5, *B. bronchiseptica* 110H; 6, *B. bronchiseptica* 110NH.

B. Western immunoblot of A with B13A8 culture supernatant.

C. Western immunoblot of A with 7H1A1 culture supernatant. Lower-case letter a, *vir*-repressed Ag-a (vra-a); lower-case letter b, *vir*-repressed Ag-b (vra-b).

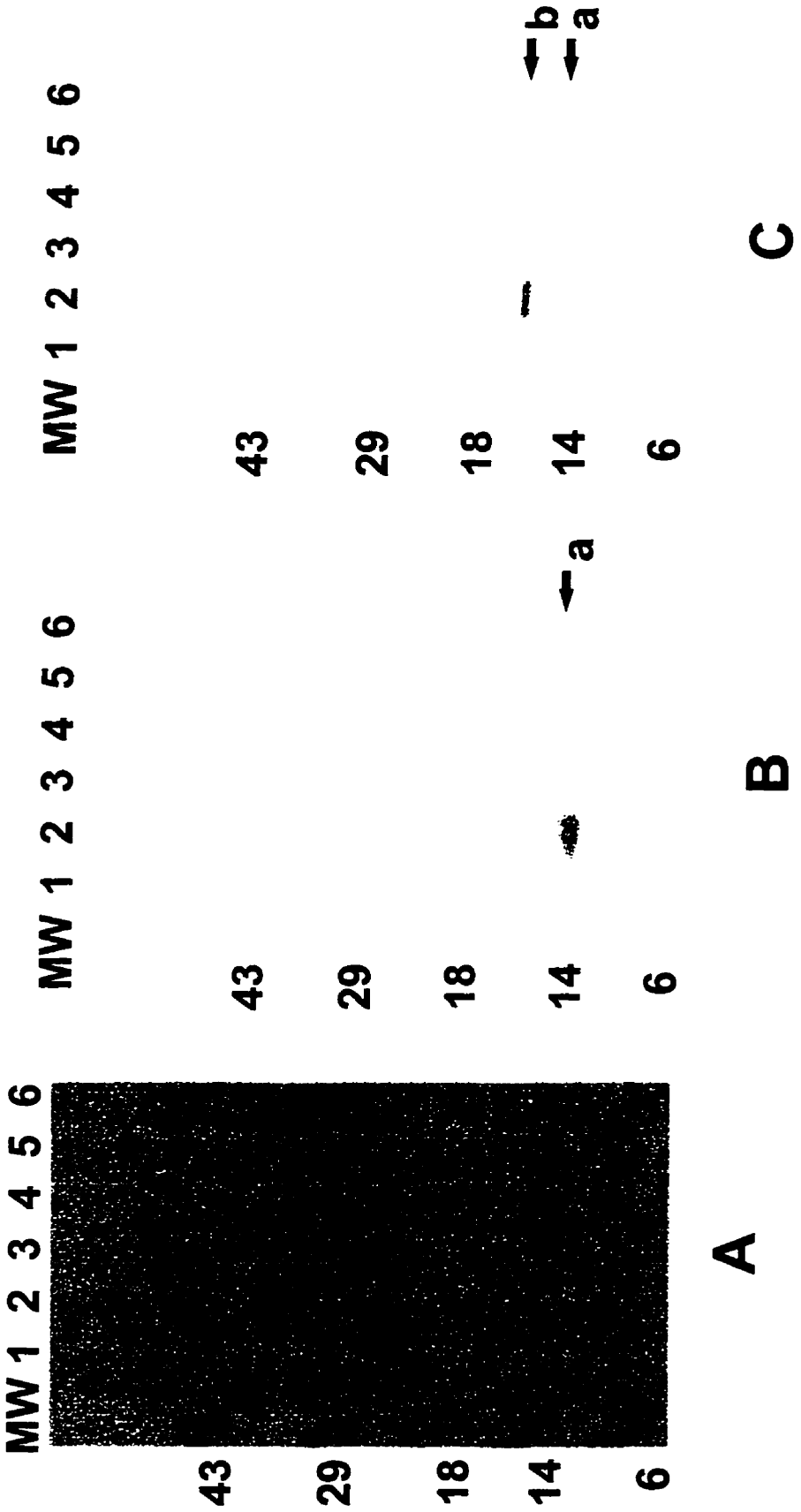

MW 1 2 3 4 5 6
43
29
18
14
6
A
MW 1 2 3 4 5 6
43
29
18
14
6
a
B
MW 1 2 3 4 5 6
43
29
18
14
6
b
a
C

FIGURE II.4

2DGE Western immunoblot analysis of *vir*-repressed antigens.

A. BP347 OMVs probed with B13A8 culture supernatant.

B. BP347 OMVs probed with 7H1A1 culture supernatant. Numbers to the right of the figure represent the apparent molecular weight of protein standards in kDa. Lower-case letter a, vra-a; lower-case letter b, vra-b.

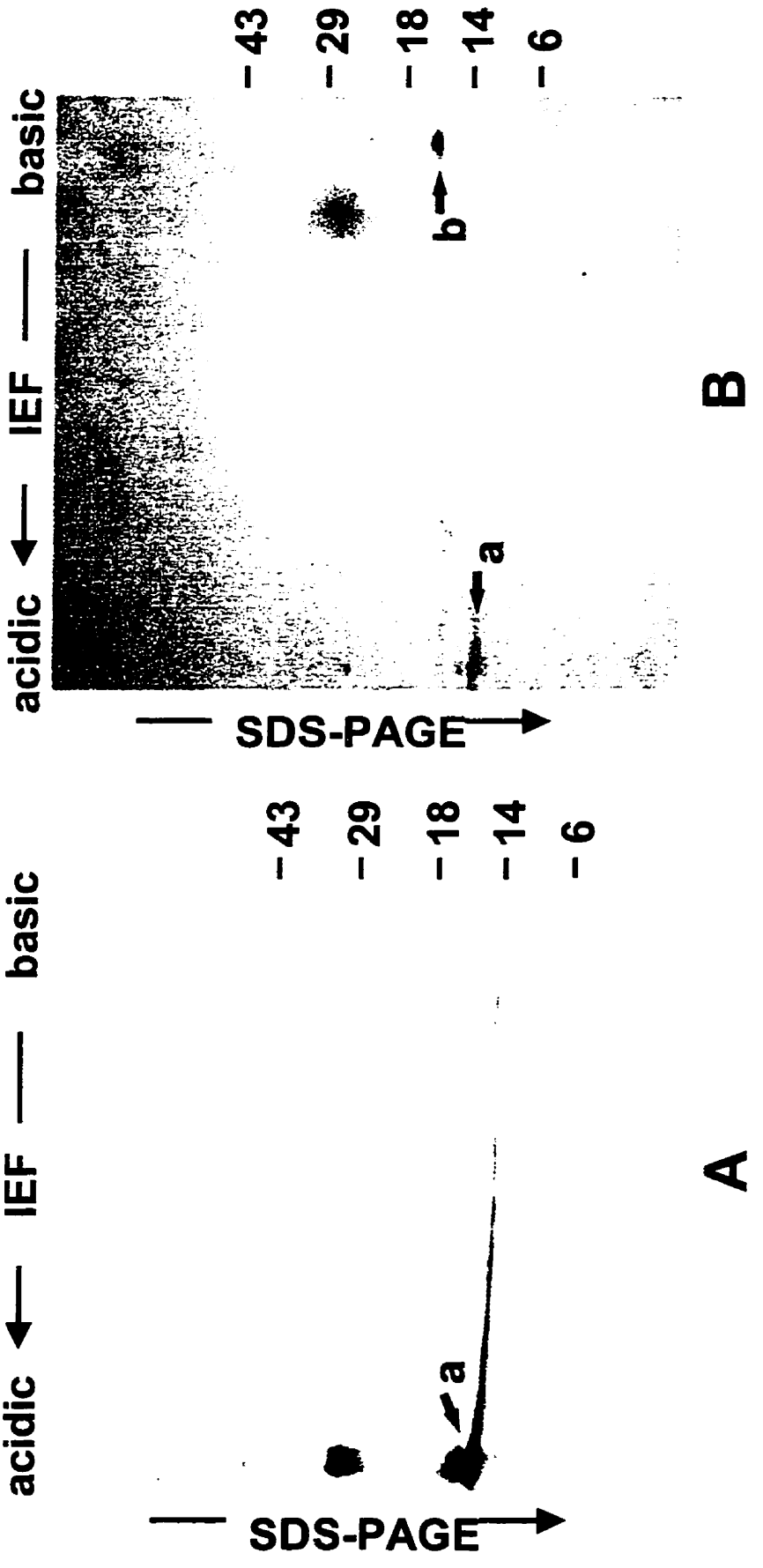

B
43
29
18
14
6
b
a
acidic
IEF
basic
SDS-PAGE
A
43
29
18
14
6
a
acidic
IEF
basic
SDS-PAGE

FIGURE II.5

Triton X-114 solubility of *vir*-repressed Ag-a and *vir*-repressed Ag-b.

A. Coomassie stained 16% SDS-PAGE. Numbers to the left of the figure represent the apparent molecular weight of protein standards in kDa. MW, Gibco-BRL protein standards; 1, BP338 OMVs Triton X-114 insoluble fraction; 2, BP338 OMVs Triton X-114 soluble fraction; 3, BP347 OMVs Triton X-114 insoluble fraction; 4, BP347 Triton X-114 soluble fraction.

B. Western immunoblot of A with B13A8 culture supernatant.

C. Western immunoblot of A with 7H1A1 culture supernatant. Lower-case letter a, vra-a; lower-case letter b, vra-b.

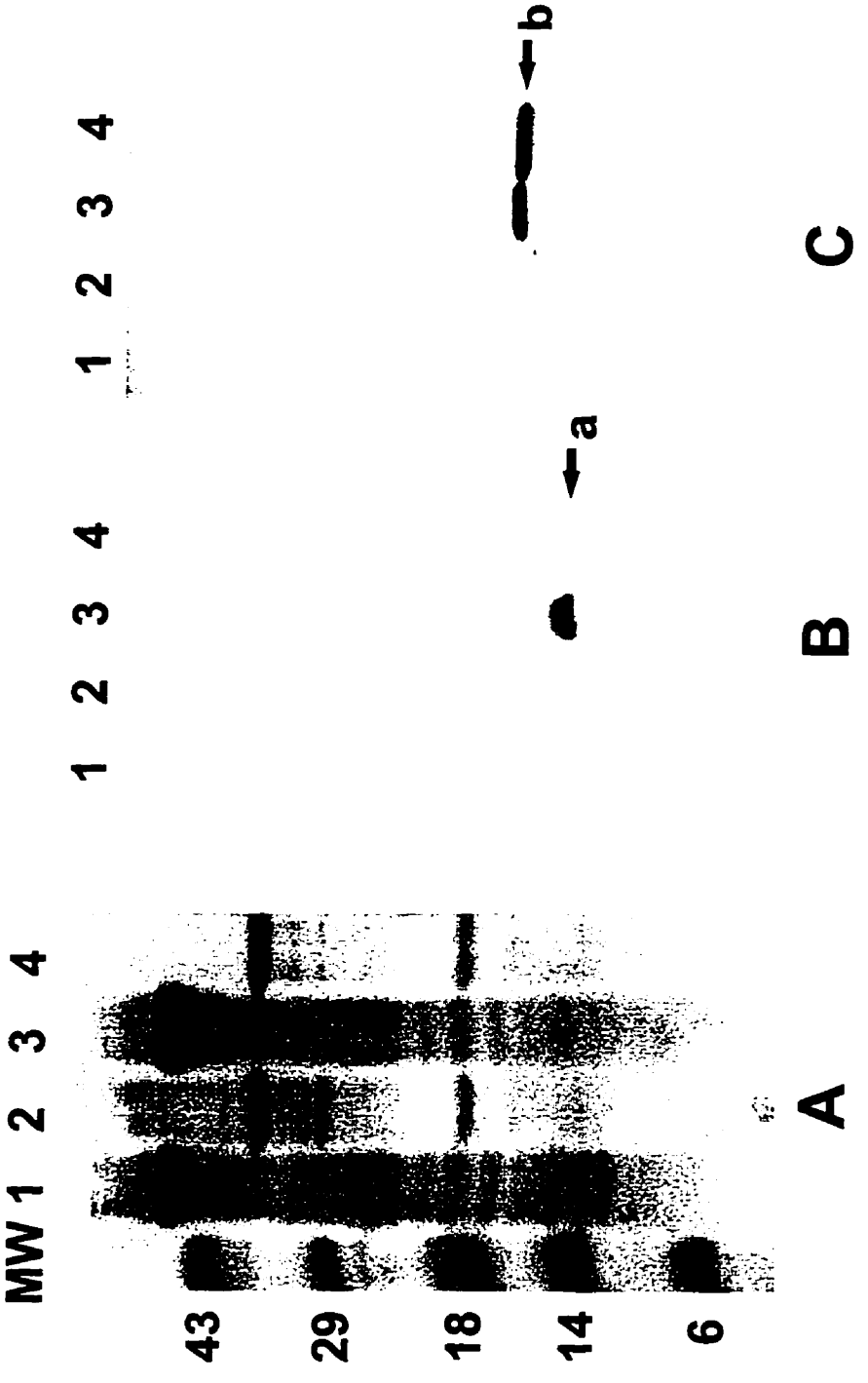

MW 1 2 3 4
43
29
18
14
6
A
1 2 3 4
a
B
1 2 3 4
b
C

FIGURE II.6.

Immunogold electronmicroscopic analysis of the phase specificity of *B. pertussis* surface Ags.

BP338 (+), virulent (phase-I) *B. pertussis* strain BP338; BP347 (-), avirulent (phase-III) *B. pertussis* strain BP347; A, BL-1, anti-LOS-A non-phase specific MAb control; B, BPE3, anti-pertactin virulent (phase-I) specific MAb control; C, 13A8B MAb culture supernatant; D, 7H1A1 MAb culture supernatant. Bar represents 1 μm.

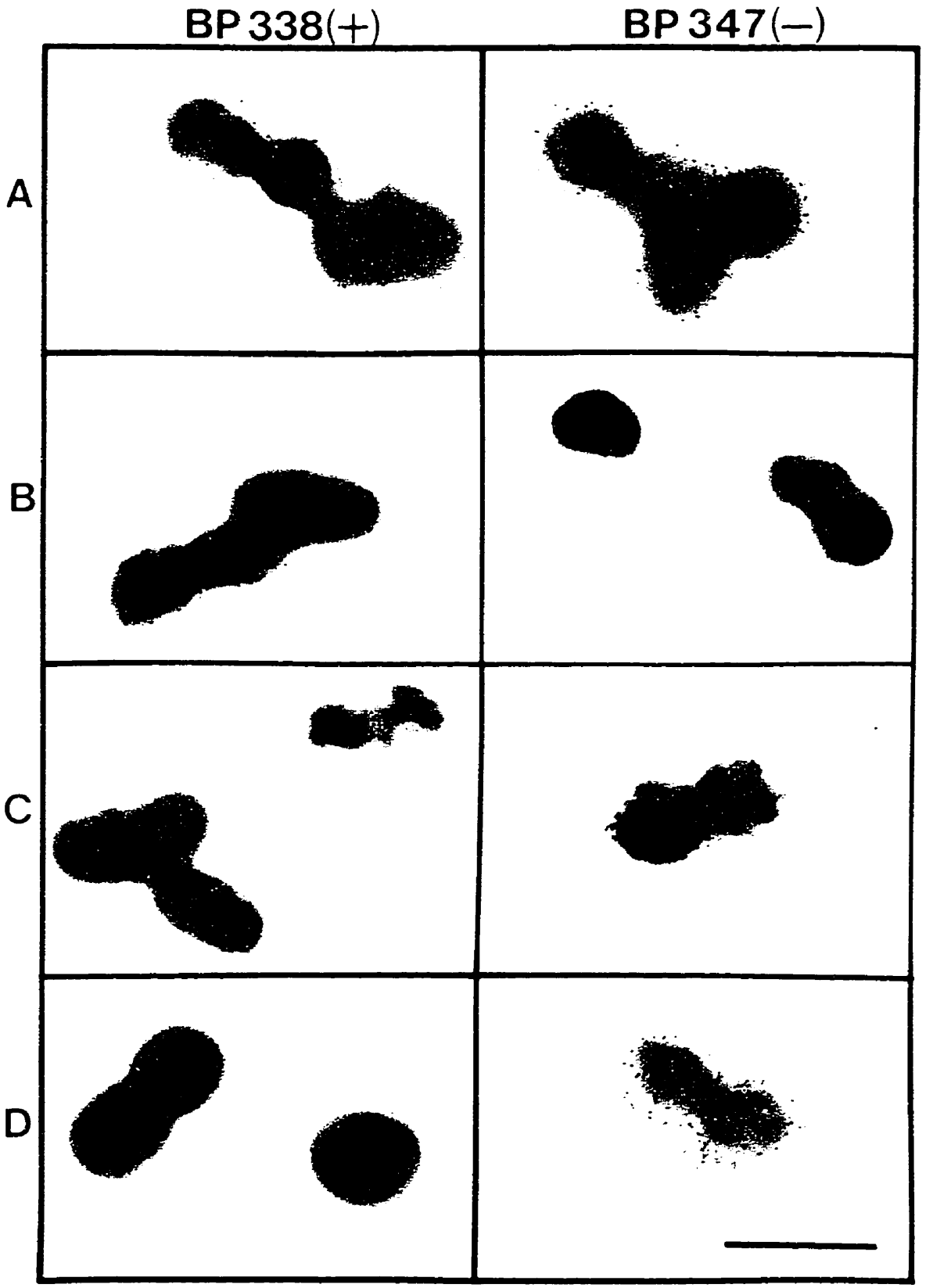

BP 338(+)
BP 347(−)
A
B
C
D

FIGURE II.7

Analysis of ^{125}I surface labeled *B. pertussis.* whole cells. I.

Autoradiogram (2 h exposure) of 15% SDS-PAGE. Numbers to the right of the figure represent the apparent molecular weight of protein standards in kDa. 1, BP338; 2, BP347. Arrows indicate labeling increased in BP347 relative to BP338.

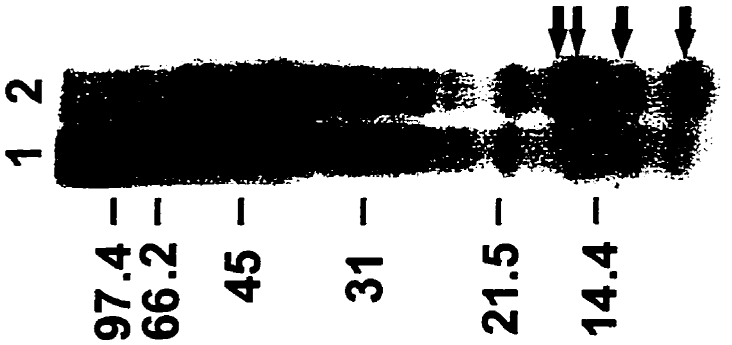

1 2
97.4
66.2
45
31
21.5
14.4

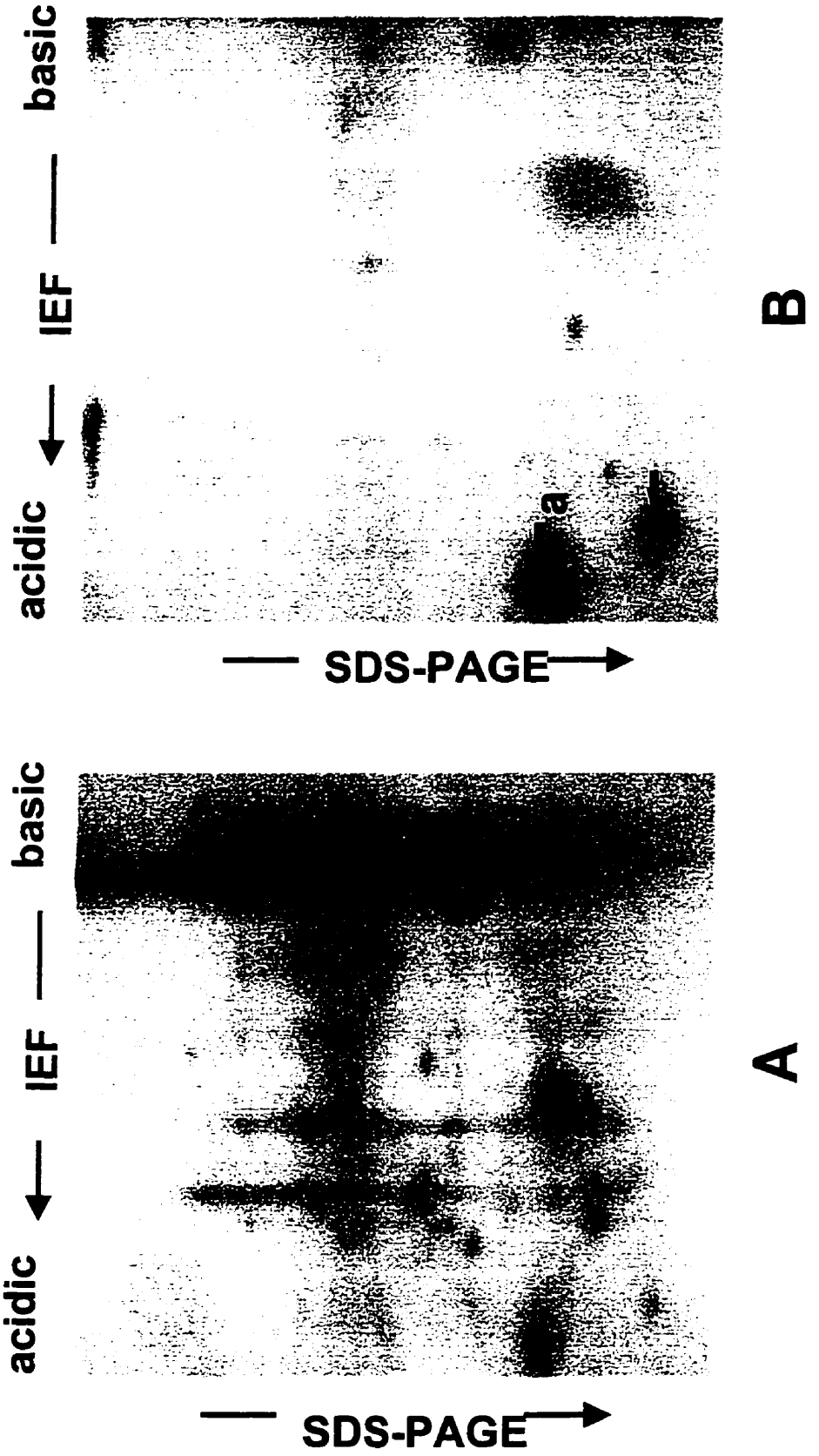
FIGURE II.8

Analysis of ^{125}I surface labeled *B. pertussis*. whole cells. II.

15% SDS-PAGE after IEF. Numbers to the right of the figure represent the apparent molecular weight of protein standards in kDa.

A. Autoradiogram (45 min exposure) of 2DGE of BP338.

B. Autoradiogram (45 min exposure) of 2DGE of BP347. Lower-case letter a, vra-a. Arrows indicate labeling increased in BP347 relative to BP338.


basic
IEF
acidic
SDS-PAGE
a
B
basic
IEF
acidic
SDS-PAGE
A

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Chapter III

DESCRIPTION OF TWO LOCI IMPORTANT FOR THE EXPRESSION OF Bvg-REPRESSED GENES AND EVALUATION OF MUTANTS IN THE HeLa CELL INVASION MODEL

A. INTRODUCTION

The etiological agent of whooping cough, *Bordetella pertussis*, expresses a number of virulence determinants which contribute to pathogenesis (58). Furthermore, the bacterium possesses the ability to coordinately regulate the expression of its virulence determinants in response to environmental stimuli through use of the two-component regulatory system BvgAS (28, 37, 53, 54, 56). When the bacterium encounters such *in vitro* signals as sulfate anions, nicotinic acid, or low temperatures, coordinate regulation results in the reduction of expression of Bvg-activated genes (*vags*) and the induction of expression of Bvg-repressed genes (*vrgs*) (8, 28, 30, 34, 41). In addition to *bvgAS*, the *vag* locus *bvgR* is necessary for regulatory repression of the *vrgs* (36). In *B. pertussis* this switching phenomenon is known as phenotypic or antigenic modulation (28, 30). While the importance of *vag*-expressing (X-mode) bacteria in *B. pertussis* pathogenesis is well established (58), the role of *vrg*-expressing bacteria (C-mode) is unknown.

In chapter I, a number of putative *vrg*-encoded proteins were described

including two (*vir*-repressed antigen-a [vra-a] and *vir*-repressed antigen-b [vra-b]) which are surface-exposed. In this chapter, further characterization of the Bvg regulation of vra-a and vra-b is described; and moreover, the importance of two putative regulatory loci, *ompR* and *acn*, in the expression of these Bvg-repressed molecules is demonstrated. Analysis of mutants in the HeLa cell invasion model demonstrates that *ompR* mutants are severely impaired in their ability to invade and survive within human epithelial-like cells; however, their adherence to these eukaryotic cells is not significantly affected. These data suggest that other regulatory molecules affect the pathways that control the regulation of the *vrgs*. Furthermore, proper expression of the *vrgs* is implicated to be crucial for invasion and survival *of B. pertussis* in a tissue culture model.

B. MATERIALS AND METHODS

1. Bacterial Strains, Plasmids, and Media.

The strains and plasmids used in this study are presented in Table III.1. *B. pertussis* strains were grown at 37°C on Bordet-Gengou agar (13) (BGA) (Difco Laboratories, Detroit, MI) containing 13% defibrinated sheep's blood (Dalynn Laboratories, Calgary, AB, Canada) in 98% humidity. To induce antigenic modulation, *B. pertussis* strains were grown on BGA containing 5 mM nicotinic acid-20 mM $MgSO_4$ (8) (BGAmod). *B. pertussis* strains were routinely grown for 3 days before use in experiments. *E. coli* strains were routinely grown

on L-agar or in L-broth (39). Where appropriate, media was supplemented with antibiotics at the concentrations: kanamycin, 25 μg/ml; ampicillin, 100 μg/ml; naladixic acid, 60 μg/ml.

2. Transposon Mutagenesis.

Triparental plate matings were used to introduce mini-Tn*5*Km into the chromosome of BP338 by a modification of the protocol of Walker and Wiess (57). *E. coli* strains grown for 3 days at room temperature (RT) were incubated at 37°C for 1 hour prior to matings. BP338 was grown on BGA for 2 days, and then streaked for confluency after overnight growth at 37°C prior to matings. *B. pertussis* was suspended to an $A_{600\ nm}$ of 1.0 in screw-capped tubes (13 by 100 mm) in Stainer-Scholte broth (49) (SSB) at 37°C, and a 1 ml aliquot used as the transposon recipient. *E. coli* conjugation donor strain C118λpir (pUT::miniTn*5*Km) and *E. coli* helper strain M294 (pRK2013) were suspended to an $A_{600\ nm}$ of 1.0 as before in LB at 37°C and 100 μl of each mixed with the *B. pertussis* recipient. The mixture was plated to 37°C Stainer-Scholte agar (SSA) containing 0.15% Bovine serum Albumin, 85 mM $MgSO_4$, and 500 μg/ml nicotinic acid. The mating plate was incubated at 37°C for 6 h, resuspended in SSB, and appropriate dilutions were plated to BGA containing appropriate antibiotics to select for exoconjugates.

3. Colony Lift Assay for Vra Expression.

After 5 days growth transposon mutagenized exoconjugates were toothpicked in duplicate to BGA and BGAmod. After 3 days growth colonies were lifted onto nitrocellulose disks (BioRad, Hercules, CA) and fixed with chloroform. Blots were blocked, probed, and developed by the same procedure as western blots described in chapter II.

4. SDS-PAGE and Western Blotting.

SDS-PAGE and western blotting were performed as described in chapter II.

5. DNA Isolation and Manipulation.

Molecular DNA manipulations were done using standard methods (3, 4, 19, 44, 45). Small-scale plasmid DNA isolation was performed by the alkaline lysis method, or with the QIAprepSpin Miniprep kit (Qiagen, Inc., Mississauga, ON, Canada). Large-scale plasmid isolation was performed using the Qiagen midi- or maxi-kits. *B. pertussis* chromosomal DNA was isolated by the CTAB (hexadecyltrimethyl ammonium bromide) method (4). DNA digests were electrophoresed in agarose gels composed of 0.6 to 1.0% agarose (BioRad) in 1X Tris-borate-EDTA buffer (44) or Tris-acetate-EDTA buffer (44, 45). Some probe labeling procedures and cloning experiments used electrophoresis in 0.8% SeaPlauque GTG low-melting point agarose (FMC BioProducts, Rockland, ME). Where necessary, probes were generated by PCR amplification using Gibco-

BRL enzymes and buffers according to the manufacturer's instructions. Probe labeling with ^{32}P was performed using random primer labeling kits according to the manufacturer's instructions. Probes were purified using size-exclusion chromatography through Sephadex-G50 (Pharmacia, Baie d'Urle, PQ, Canada) columns in TE buffer (44). Southern blotting to nitrocellulose was performed by standard techniques (19, 44, 48). Pulsed-field gels containing large DNA fragments were dupurinated in 0.25 M HCl for 15 min before processing for Southern transfer. Colony hybridization to nylon membranes (Hybond-C; Amersham Life Sciences, Arlington Hts., IL) was performed by standard techniques (44).

6. DNA Hybridizations.

Prior to high-stringency hybridization (19, 44), membranes were wet in 4X SSPE (0.18 M NaCl-10 mM NaH_2PO_4-1 mM EDTA, pH 7.2 [1X SSPE]). Prehybridization of membrane-bound DNA was performed in 50% formamide-2.5X Denhardt's solution (2% Ficoll-2% polyvinylpyrrolidone-2% bovine serum albumin [100X Denhardt's solution])-0.5% sodium dodecyl sulfate-100 μg/ml salmon sperm DNA-5% dextran sulfate-0.625X SSPE at 42°C. High-stringency hybridization of was performed in 50% formamide-0.5X Denhardt's solution-0.5% sodium dodecyl sulfate-100 μg/ml salmon sperm DNA-5% dextran sulfate-0.625X SSPE at 42°C. Membranes were washed in 0.2X SSPE-0.1% sodium dodecyl sulfate at 56°C. Hybridizing DNA was detected by autoradiography using Kodak

X-Omat-R film (Eastman Kodak Co., Rochester, NY).

7. Molecular Cloning and DNA Sequence Analysis.

Standard cloning techniques were applied using restriction enzymes according to the manufacturer's instructions. Routine cloning and subcloning was done in E. coli DH5α or XL-1 blue using pBSKS-. Where appropriate vectors were treated with shrimp alkaline phosphate (SAP) (United States Biochemical, Arlington Hts., IL) according to the manufacturer's instructions. Purification of DNA from agarose gels was performed using the QIAEXII Gel Extraction kit (Qiagen). Alternatively, DNA was purified from SeaPlaque GTG low-melting point agarose using Agarase (New England Biolabs, Mississauga, ON, Canada) according to the manufacturer's instructions. Ligations were performed using T4 ligase (Gibco-BRL, Burlington, ON, Canada) according to the manufacturer's instructions. Preparation and transformation of competent cells was performed by standard techniques (27, 44). DNA sequencing was performed using the ThermoSequencing kit (Amersham). Alternatively, sequencing was performed by the DNA core facility (Dept. of Biochemistry, Univ. of Alberta, AB, Canada). Oligonucleotides were purchased from the DNA core facility, or BioServe Biotechnologies Ltd., Laurel, MD. DNA sequence comparisons were performed using the basic local alignment search tool (BLAST) algorithm (1) via the National Center for Biotechnology WWW server (http://www.ncbi.nlm.nih.gov).

8. Electroporation of *B. pertussis*.

Electroporation of *B. pertussis* was performed by the method of Zealey *et al.* (62). BGA grown *B. pertussis* strains were suspended in SSB to an $A_{540\ nm}$ of 1.0 as before and diluted 1 in 25 in SSB. Broth cultures were grown at 37°C with shaking at 150 rpm for 3 days, and then pelleted by centrifugation at 12 000 x *g* at 4°C. Cells were washed twice by centrifugation in 500 ml double-distilled H_2O, and once in 50 ml 272 mM sucrose-15% glycerol. Frozen electrocompetent cells were prepared by resuspension in 10 ml 272 mM sucrose-15% glycerol and flash-freezing using liquid N_2. 10 µl Salt-free DNA was transformed using a BioRad pulse controller using 600 µl electrocompetent cells at 4°C, and an electrode gap of 0.2 cm on a BioRad pulse controller with the settings: voltage, 2.5 kV; capacitor, 25 µF; pulse, 400 ohms (10 msec). After transformation, cells were outgrown in 5 ml SSB with shaking at 150 rpm for 1 h at 37°C, harvested by centrifugation, and then plated to BGA with appropriate antibiotics.

9. Pulsed-Field Gel Electrophoresis (PFGE).

PFGE of *B. pertussis* chromosomal DNA was performed essentially by the method of de Moissac *et al.* (16) using a modification of the embedding technique of Smith and Cantor (47). Bacteria were suspended in to an $A_{540\ nm}$ of 0.6 as before in 50 mM Tris-HCl-5-mM EDTA, pH 8.0 (TE) and 1 mL aliquots pelleted by centrifugation in a microfuge (approximately 10 000 x *g*) for 5 min.

Pellets were resuspended in 250 µl of 10 mM $MgCl_2$, vortexed with 250 µl of liquid 2% low-melting point agarose (UltraPURE; Gibco-BRL) in 0.5X Tris-borate-EDTA (500 mM Tris-borate-10 mM EDTA [5X TBE]), and pipetted into molds to create agarose plugs. Processing of the plugs was done in volumes of 3 ml. Plugs were incubated in lysis buffer (0.1 M EDTA [pH 8.0]-0.01 M Tris-HCl [pH 7.6]-0.5% sodium dodecyl sulfate-0.5% sodium *N*-lauroyl sarcosine-0.2% deoxycholic acid-200 µg/ml RNAse-1 mg/ml lysozyme) overnight at 37°C and then transferred to SE buffer (1% sodium *N*-lauroyl sarcosine-0.5 M EDTA [pH 8.0]) containing 1 mg/ml proteinase K. After incubation at 50°C for 16 to 18 h, plugs were incubated in 2 changes of TE-0.1 mM PMSF (phenylmethylsulfonyl fluoride) for 45 min each at RT. Plugs were then incubated in TE for 1 h at RT, placed in fresh TE, and then incubated for 16 to 18 h at 37°C. Plugs containing the released DNA were stored in TE at 4°C when not digested immediately. The DNA was digested using *Xba*I or *Spe*I according to the manufacturer's instructions using gel slices of an approximate volume of 30 µl in 200 µl total volume reaction mixtures. DNA was digested overnight at 37°C and the reaction stopped by addition of 200 µl of 0.5 M EDTA (pH 8.0). Plug slices were loaded onto 1% agarose (KILOrose; Clonetech Laboratories, Inc., Palo Alto, CA) gels cast in 0.5X TBE, and electrophoresed in 0.5X TBE using a CHEF-DRIII apparatus (BioRad) with the run parameters: initial pulse time, 5 s; final pulse time, 45s; 5.6 V/cm; 14°C; run time, 14 h. Sizing of bands was done by comparison with DNA standards (Lambda Ladder PFG Marker; New England

Biolabs). Alternatively, using a modified protocol of Stibitz and Garletts (52), gels were cast and run in 0.5X pulsed-field TBE (75 mM Tris-25 mM boric acid-0.1 mM EDTA [1X p.f. TBE]) with the run parameters: initial pulse time, 1 s; final pulse time, 50s; 5.2 V/cm; 5°C; run time, 28 h.

10. Chromosomal Mapping.

Chromosomal mapping was performed by Southern hybridization following pulse-field gel electrophoresis. Alternatively, chromosomal mapping of one locus, *acn*, was facilitated by insertion of *Xba*I and *Spe*I sites into the *B. pertussis* chromosome via homologous recombination using the method of Stibitz and Garletts (52). Briefly, an *acn* fragment was cloned into pSS1577 to create pT1a, which was then transformed into *E. coli* strain S17.1. The plasmid was mated into BP536 using biparental matings done exactly as for triparental matings described previously, except only one *E. coli* strain, S17.1, was used for conjugations. Exoconjugates were selected on BGA containing appropriate antibiotics. Pulsed-field gel electrophoresis of chromosomal DNA reveals additional *Xba*I and *Spe*I fragments at the locus of interest and facilitates mapping to the *B. pertussis* Tohama I physical chromosomal map (50, 52).

11. HeLa Cell Invasion Assay.

The gentamycin invasion assay was performed essentially by the method of Ewanowich *et al.* (20). 24-well tissue culture trays with greater than 90% confluent HeLa cell monolayers (human epithelial-like; ATTC CCL 2.1) were

used in the invasion assays. *B. pertussis* was suspended to an $A_{540\ nm}$ of 0.12 as before in Eagle minimal essential medium (MEM; Gibco-BRL) supplemented with 3% FBS (Flow Laboratories, McLean, VI). Bacterial suspensions were diluted 1 in 25 in MEM-3% FBS and 400 µl was added per well (approximately 9 x 10^6 CFU). Tissue culture trays were incubated statically at 37°C in 5% CO_2. After 5 h of incubation the wells were washed 4 times with 500 µl MEM, and the media replaced with 400 µl MEM-3% FBS containing 100 µg/ml gentamycin. After 2 h incubation at 37°C in 5% CO_2 the monolayers were washed 4 times with 500 µl MEM, and then rinsed with 500 µL FC wash (25 mM Tris-137 mM NaCl-5.37 mM KCl-O.56 mM EDTA, pH 7.0). Monolayers were then treated with 0.25% trypsin-FC wash to lift the cells, diluted in MEM-3% FBS, and plated to BGA in triplicate for determination of intracellular CFUs.

12. HeLa Cell Adherence Assay.

The relative adherence of *B. pertussis* strains to HeLa cells was performed in 24-well tissue culture trays with greater than 90% confluent HeLa cell monolayers. Strains were added to wells as in the invasion assay in MEM-3% FBS, and the trays incubated at 37°C in 5% CO_2 for 90 min. Trays were then removed and washed 5 times in 500 µl MEM for 5 min with rotation at 60 rpm at room temperature using 500 µl MEM-3%FBS. Monolayers were then rinsed with FC wash, treated with 0.25% trypsin-FC wash to lift the monolayers, diluted in MEM-3% FBS, and plated to BGA in triplicate for determination of total CFUs.

C. RESULTS

1. Expression of vras in BvgS constitutive mutants.

Bvg+ strain BP370 and its BvgS constitutive derivatives SJ301, SJ303, and SJ304 were tested for their ability to express vras. BP370 produces domed hemolytic colonies on BGA and flat non-hemolytic colonies on BGAmod confirming its virulent phenotype (Bvg+) and ability to modulate. Strains SJ301, SJ303, and SJ304 produce domed hemolytic colonies on both BGA and BGAmod confirming their constitutive virulent phenotype (BvgS-c). Western blots probed simultaneously with anti-vra-a and anti-vra-b MAbs demonstrate that only the parental strain BP370, and not its BvgS-c derivatives, produce increased amounts of the vras on modulating media (Fig. III.1).

2. Expression of vras in *bvgR* mutants.

Bvg+ strain TM1081 and its *bvgR* mutant derivatives TM1081-T1, TM1081-T16, TM1126, and TM1210 were tested for their ability to express vras. TM1081 and its derivatives produce domed hemolytic colonies on BGA and flat non-hemolytic colonies on BGAmod confirming their ability to express a virulent-like phenotype (BvgAS+) and their ability to modulate. Western blots probed with anti-vra-a or anti-vra-b MAbs demonstrate that the parental strain TM1081 produces increased amounts of the vras on BGAmod relative to BGA, while the *bvgR* mutant derivatives constitutively produce both vra-a and vra-b (Fig. III.2A and Fig. III.2B).

3. Expression of vras in two mini-Tn*5* transposon mutants.

Using transposon mutagenesis, a bank of BP338 derivatives were constructed and screened with anti-vra-b and anti-vra-a MAbs using a colony lift assay. Of over five thousand exoconjugates colonies screened in this manner only two, DLM20 and DLM21, showed no reactivity above background levels with anti-vra antibodies after growth on either BGA or BGAmod (data not shown). The other exoconjugates colonies behaved like wild-type *B. pertussis* reacting above background with the anti-vra antibodies when grown on BGA mod, but not when grown on BGA. BP338 control colonies produce domed hemolytic colonies on BGA and flat non-hemolytic colonies on BGAmod confirming its virulent phenotype (Bvg+) and its ability to modulate. BP338 colonies grown on BGAmod, but not those grown on BGA, react to the anti-vra MAbs above background levels. BP347 control colonies are flat and non-hemolytic on both BGA and BGAmod confirming the strains constitutive avirulent (Bvg-) phenotype. BP347 colonies react with anti-vra MAbs above background levels when grown on either BGA or BGAmod. DLM20 and DLM21 colonies are domed and hemolytic on BGA and flat and non-hemolytic on BGAmod confirming their virulence phenotype, and their ability to modulate. No differences were observable between the modulated or unmodulated protein profiles of DLM20 and DLM21 compared to these profiles in parental strain BP338 by SDS-PAGE (data not shown). Western blots probed with anti-vra-a or anti-vra-b MAbs demonstrate that the parental strain BP338 produces increased amounts of vra-a and vra-b when grown on BGA mod relative to growth on BGA (Fig. III.3A and

Fig. III.3B). Strain DLM20 does not produce amounts of vra-a or vra-b greater that those produced by BP338 grown on BGA, regardless of the growth medium, and thus vra induction is seen to be absent in DLM20. Strain DLM21 shows decreased reactivity with the anti-vra MAbs when grown on BGAmod compared to BP338 reactivity when grown on BGAmod, while the amounts of expression are the same (low or absent) for each strain when grown on BGA. DLM21 thus has a reduced ability to produce vras when grown on modulating media.

4. Southern hybridization analysis of DLM20 and DLM21.

DLM20 and DLM21 have similar phenotypes with respect to their ability to induce vra expression when grown on modulating media. To test if the transposon insertions in these mutants are in similar locations, southern analysis using a Tn*5* probe derived from a 2 kb *Sfi*I fragment of pUT::mini-Tn5Km was performed. Hybridizing fragments from DLM20 and DLM21 chromosomal DNA, detected after digestion with six separate restriction enzymes and electrophoresis in 0.8% agarose, show unique banding patterns (Fig. III.4). In addition, only one hybridizing band is seen in each digest lane for both strains demonstrating the mutants to be the result of unique single insertions of Tn*5* in their chromosomes.

5. Cloning and partial sequencing of Tn*5* flanking regions.

Size-selected, single restriction enzyme digested chromosomal DNA from strains DLM20 and DLM21 were used to clone transposon containing fragments

from each strain. Chromosomal DNA fragments were ligated into SAP-treated, restriction enzyme digested pBSKS-, transformed into *E. coli*, and doubly selected for kanamycin and ampicillin resistance. Clones, p21ClaI (~ 5 kb insert) and p21EcoRV (~ 7 kb insert) were generated from DLM21 in this manner, while clones p20XhoI (~ 7 kb insert) and p20NotI (~ 3 kb insert) were generated from DLM20. Partial sequencing of the DNA inserts using pBSKS- specific and mini-Tn*5*Km specific primers, and primer walking, reveal Tn*5*-flanking DNA which is highly homologous to the *ompR* family of transcriptional regulators for DLM20, and DNA homologous to the aconitase (*acn*) family of regulators for DLM21 (table III.2 and table III.3, respectively). Using BLASTN analysis, a 612 bp segment of DLM20 Tn*5*-flanking DNA produces high score segment pairs of 426 with *N. meningitidis ompR* (60) with smallest sum probability numbers of $p(N) = 4.1e^{-25}$. Sequence comparisons with the unpublished sequence of *B. pertussis ompR* (32) reveals identity with the DLM20 Tn*5*-flanking region, and furthermore reveals the site of Tn*5* insertion to be between nucleotides 632 and 633 of the *ompR* sequence. Using BLASTN analysis, an 812 bp unambiguous (both strands sequenced) segment of DLM21 Tn*5*-flanking DNA produces high score segment pairs of 739 with *Xanthomonas campestris rpfA* (61), and a high score segment pairs of 708 with *Bradyrhizobium japonicum acn* (55), with smallest sum probability numbers of $p(N) = 1.5e^{-113}$ and $p(N) = 6.1e^{-108}$, respectively.

6. Expression of vras in a *B. pertussis ompR* mutant.

Bvg+ strain BP536 and its *ompR* mutant derivative OMPR- were tested for their ability to express vras by western blot analysis. BP536, and OMPR- produce domed hemolytic colonies on BGA, and flat non-hemolytic colonies on BGAmod confirming their virulent phenotype (Bvg+) and ability to modulate. No differences were observable between the modulated or unmodulated protein profiles of OMPR- compared to these profiles in parental strain BP536 by SDS-PAGE (data not shown). Western blots probed simultaneously with anti-vra-a and anti-vra-b MAbs demonstrate that only the parental strain BP536, and not OMPR-, produce increased amounts of the vras on modulating media (Fig. III.5).

7. Transformation of DLM21 with PBBR1MCS plasmids.

Size-selected, *Sau*3A1 partially digested chromosomal DNA from strain BP338 was used to construct a plasmid library of *B. pertussis* chromosomal DNA by ligation into SAP-treated, enzyme digested pBBR1MCS-4, which was transformed and selected for by ampicillin resistance in *E. coli*. Clones were screened by colony hybridization with a 649 bp *acn* probe generated by PCR using primers designed from the partial sequence of the putative *B. pertussis acn* gene. Two positive clones, p9.4 (~ 3.2 kb insert) and p10.1 (~ 4.5 kb insert) were isolated, and their inserts partially sequenced after subcloning into pBSKS- to confirm their identity with the putative *B. pertussis acn* gene (data not shown). pBBR1MCS-4, p9.4, and p10.1 were each electroporated into BP338 and DLM21 and the resulting strains were tested for their ability to express vras by western

blot analysis. The transformed strains produce domed hemolytic colonies when grown on BGA, and flat non-hemolytic colonies when grown on BGAmod confirming their virulent phenotype (Bvg+) and ability to modulate. Western blots probed simultaneously with anti-vra-a and anti-vra-b MAbs demonstrate that the strains DLM20 (pBBR1MCS-4), DLM21 (p9.4) and DLM21 (p10.1) produce decreased amounts of vra-a and vra-b when grown on BGAmod relative to BP338 (pBBR1MCS-4), BP338 (p9.4), and BP338 (p10.1) grown on BGAmod, indicating that the mutant phenotype of DLM21 was not complemented. (Fig. III.6). Sequence analysis of the inserts of p9.4 and p10.1 using BLASTN reveal that the promoter of the putative *acn* is not likely present (data not shown). Furthermore, aconitase genes are large (often greater than 3.5 kb) and thus the entire gene was not likely present in the pBBR1MCS-4 inserts.

8. Chromosomal mapping.

To map the position of Tn*5* insertion into strains DLM20 and DLM21, southern analysis after pulsed-field gel electrophoresis of chromosomal DNA using a Tn5 probe derived from a 2 kb *Sfi*I fragment of pUT::mini-Tn*5*Km was performed. Banding patterns reveal that in DLM2O the probe hybridizes to fragments corresponding to Spe C and Xba H of the Tohama I physical map (52, 61), while in DLM21 the probe hybridizes to fragments Spe D and the position of fragments Xba L1, L2, L3, and L4 (Fig. III.7). Based on the published map this position must correspond to Xba L1 or L2 in DLM21. The identity of the disrupted gene as *ompR* is thus further established as *ompR* has been fine-mapped to

fragments Spe C and Xba H. As expected, BP338 does not hybridize with the Tn*5* probe, while strain BP347 (Tn*5*::*bvgS*) produces hybridizing bands corresponding to Spe D and Xba L1 (Xba L1-L4) in agreement with the published map. Chromosomal mapping of the putative aconitase gene was refined using a 4.5 kb fragment of the p10.1 insert cloned into pSS1577 to create pT1a, which was shuttled into the BP536 chromosome to generate strain TS11a. Pulsed-field gel electrophoresis of strain TS11a reveals that the Spe D fragment is missing, while the Xba L fragments appear less intense and smaller relative to those produced by the parental strain BP536 (Fig. III.8). Closer inspection reveals that the Spe E band (Spe E1, E2, and E3) is slightly larger and more intense than that seen in BP536. In addition, a new band appears in *Xba*I digest between the Xba M and Xba N bands. These data indicate that the putative *B. pertussis* aconitase gene is within Spe D and Xba L2, at a position approximately in the middle of Xba L2 in the *B. pertussis* chromosome (Fig. III.9).

9. Invasion of HeLa cells by Tohama I derivatives.

The invasive ability of the transposon mutants DLM20 and DLM21, as well as strain OMPR- were tested using the HeLa cell-gentamycin invasion assay. DLM20 has a reduced ability to enter and survive in HeLa cells relative to the parental strain BP338 with about 20% of CFUs recovered per well ($P < 0.01$, Student's t-test). Total numbers of invasive BP338 CFUs per well agree with those previously published (20). Control non-invasive strain BP347 produces about 0.5% of CFUs per well relative to BP338 ($P < 0.01$) in accordance with

published data (20). DLM20 is significantly more invasive than BP347 ($P < 0.01$). DLM21 invasive ability is not significantly different than that of parental BP338 ($P > 0.25$). These data are summarized in figure III.10. Strain OMPR- has a reduced ability to enter and survive relative to the parental strain BP536 with about 15% of CFUs per well ($P < 0.01$) as illustrated in figure III.11.

10. Adherence to HeLa cells by Tohama I derivatives.

To test whether the reduced ability of DLM20 and OMPR- to invade HeLa cells was the result of an initial adherence defect, HeLa adherence assays were performed. After 90 min incubation and washing to remove non-adherent bacteria, DLM20 shows no significant difference in adherence to HeLa cell monolayers ($P > 0.25$) relative to the parental strain BP338. DLM21 shows a slight reduction of adherence relative to BP338 (about 80% of CFUs, $P = 0.13$). Avirulent control strain BP347 is much less adherent relative to parental strain BP338 with about 1.5% CFUs recovered per well ($P < 0.01$), and is significantly less adherent than both DLM20 and DLM21 ($P < 0.01$). These data are summarized in figure III.12. OMPR- shows no significant difference in adherence to HeLa cell monolayers relative to its parental strain BP536 ($P > 0.25$). as illustrated in figure III.13.

D. DISCUSSION

The ability to coordinately regulate gene expression is common to a number of bacterial pathogens (2, 18, 38). Moreover, this ability is known to be crucial for the virulence and survival of bacteria pathogens in response to the varied and changeful environments encountered within and without their natural hosts (18, 21). In the upper-respiratory pathogen *Bordetella pertussis*, global regulation of gene expression occurs as result of the complex BvgAS two-component regulatory system (53, 56). *B. pertussis* produces a large number of virulence factors that contribute to pathogenesis, and the majority of the encoding genes (*vags*) are subject to activation by BvgAS (53, 58). It is also clear that *B. pertussis* expresses another set of genes which are under the regulatory control of BvgAS, but expressed reciprocally to the Bvg-activated virulence factors (10, 28). The role of these Bvg-repressed genes (*vrgs*) and their products in the life-cycle of *B. pertussis* is unknown, and in comparison to the Bvg-activated genes and products they are not well characterized. The experiments presented in this chapter sought to further characterize the regulation of the Bvg-repressed surface proteins vra-a and vra-b, with the tenet that a better understanding of their regulation may give clues as to their potential role in pathogenesis or survival of the *B. pertussis*.

The work of Miller *et al.* (37) established BvgAS as responsible for the environmental response to *in vivo* modulating agents by isolation of a series of bacteria (with mutations in the BvgS linker domain) that are unresponsive to

modulation. Unlike bacteria with gross mutations in BvgAS (43, 54, 59), these BvgS-constitutive, point-mutated bacteria, still produce the Bvg-activated factors but their expression is not repressed by modulating agents. On the basis of the coordinate model, expression of *vrgs* in these mutants are constitutively repressed, and thus not induced by the presence of modulating agents. To further establish the proteins vra-a and vra-b as being under the regulatory control of BvgAS, and further establish them as likely products of *vrgs*, we tested BvgS-constitutive strains for their ability to express the vras under non-modulating and modulating conditions. Unlike the parental strain, the BvgS-constitutive strains do not show induction of the vras under modulating conditions and produce a constitutive repressed phenotype. As expected from work described in chapter I, this demonstrates that the vras are under the regulatory control of BvgAS.

The work of Beattie *et al.* (8-10) postulated that regulatory repression of the *vrgs* is due to binding of Bvg-activated repressor to an intragenic sequence which has been found in four of the five characterized *B. pertussis vrgs*. Merkel and Stibitz (36) further refined this paradigm by isolating a series of repressor mutants which map just downstream of *bvgAS* in a locus named *bvgR*. Mutations in this locus result in the constitutive expression of *vrgs* including *vrg-73* for which no putative repressor binding site has yet been identified, while *vag* activation is unaffected. To definitively establish vra-a and vra-b as being under the regulatory control of the BvgASR regulatory cascade, and to again establish them as likely products of *vrgs*, we tested *bvgR* mutants for their ability to

express the vras under non-modulating and modulating conditions. The parental strain, shows normal vra derepression under modulating conditions, while all the *bvgR* mutants display constitutive expression of the vras regardless of the growth medium. Hence, vra-a and vra-b are under hierarchical control of the *bvgR* locus; establishing BvgR as a putative master repressor of the *vrgs* and their products. Cloning and sequencing of the structural genes for vra-a and vra-b will be required to definitively establish whether they are *vrg*-products under the direct control of BvgR, or if they are themselves regulated by a *vrg* or *vrgs*, and thereby are indirectly Bvg-repressed.

Although BvgAS is well established as the master regulon of *B. pertussis* virulence gene expression, there may be other regulators and signals that affect the expression of these regulons. Indeed, the presence of multiple regulatory systems and signals which affect gene regulation and pathogenesis is common to a number of pathogens (18, 21). Using transposon mutagenesis, a series of mutants were created and tested for alterations in expression of vra-a and vra-b. Two of these, DLM20 and DLM21, each have decreased expression of both vra-a and vra-b. Modulated DLM20 shows no induced expression of these proteins, while modulated DLM21 shows lowered expression of these proteins relative to its modulated parental strain. Southern analysis demonstrated that the phenotypes of these mutants are the result of single unique insertions of Tn*5* in their chromosomes. Furthermore, although they have similar vra phenotypes, chromosomal mapping demonstrates that the affected loci are in entirely different

locations on the *B. pertussis* chromosome, and thus they are not physically linked.

Cloning and partial sequencing of Tn*5* flanking regions established the mutagenized locus in DLM20 to be *ompR*. Furthermore, a specific *ompR* mutation was seen to result in the same vra phenotype. This mutant is known to express *vag* loci normally (32) raising the possibility that the *vrgs* are specifically under the regulatory control of OmpR. In *E. coli* and other Gram-negative organisms the EnvZ-OmpR two component regulatory system controls the expression of genes, including the porin genes *ompC* and *ompF,* in response to changes in osmolarity via a classical two-component phosphorelay cascade (40). However, in all *B. pertussis* strains (including Tohama I derivatives) tested so far, the *envZ* locus encoding the osmosensor appears to be silent (32). Thus, if OmpR is acting as a transactivator of gene expression resulting in regulated expression of the *vrgs*, its activity must be itself regulated by some molecule other than EnvZ. One intriguing possibility is that the activating molecule is BvgS. BvgS is known to be a complex sensor molecule with several domains (25, 40) which perhaps act as potential signal input and output switches to activate molecules other than BvgA. Cross-talk of regulatory systems resulting in the activation of OmpR has been postulated for the intestinal pathogen *Shigella flexneri* (11). Analysis of a *vir::lac* fusion within an *S. flexneri* invasion gene showed that the gene was regulated in an osmodependent fashion by EnvZ-OmpR. A mutation in EnvZ resulted in a loss of virulence and the inability to invade HeLa cells; however, expression of the *vir::lac* fusion still responded in an

osmodependent manner suggesting that activation of OmpR by regulators other than EnvZ could exist. Of course, the possibility exists that OmpR activity is regulated by a pathway distinct from Bvg, or by several unknown pathways.

It will also be interesting to determine whether OmpR acts directly with the upstream regions of the *vrgs* to regulate expression at the level of transcription. One model for such regulation can be hypothesized by consideration of the mechanism of OmpR regulation of *ompF* in *E. coli* (40). In *E. coli*, low concentrations of phosphorylated OmpR favor transcription activation of *ompF* through binding of specific upstream regions. However, higher concentrations of phosphorylated OmpR result in binding to additional upstream regions and acts to repress transcription. If such a mechanism exists in *B. pertussis* for the *vrgs*, then an OmpR null mutation would result in reduced transcription (as is seen in DLM20 and OMPR-) as the activator would not be present. However, increased concentrations of phosphorylated OmpR following its activation could act to repress *vrg* transcription via OmpR binding to additional *vrg cis*-acting sites. Once again, many other possibilities for both indirect and direct activation of the *vrgs* can be envisioned, and further work to determine the complexity of *vrg* regulation in *B. pertussis* should prove intriguing.

Using *phoA*-transcriptional fusions in DLM20, it has been shown that *vrg-6* and *vrg-73*, like vra-a and vra-b, do not show induced expression on modulating media (35). Hence, mutation of *ompR* appears to affect the entire *vrg* regulon. It is not clear at present whether genes other than *vrgs* are affected by *ompR* mutation. The *vags* appear not to be effected however, as both DLM20 and

OMPR- colonies display a morphology consistent with normal *vag* expression (domed and hemolytic on BGA), and the DLM20 one-dimensional SDS-PAGE protein profiles appear unchanged. Furthermore, normal regulation of *vag* expression in OMPR- has been reported (32), and *vag* products of DLM20 appear to be regulated normally as demonstrated by western blotting (35). It will also be interesting to see whether porin genes, such as the Bvg-activated porin-like gene *ompQ* (22) are affected by OmpR regulation. The possibility exists that OmpR acts globally to affect regulation of many genes.

Cloning and partial sequencing of Tn*5* flanking regions established the mutagenized locus in DLM21 to be an aconitase-like gene (*acn*) of the IRP (iron-repressive protein)-aconitase family (23). Using *phoA*-transcriptional fusions in DLM21, it has been shown that *vrg-6* and *vrg-73*, like vra-a and vra-b, show reduced expression on modulating media relative to its parent BP338 (35). Hence, like the *ompR* mutation in DLM20, the *acn* mutation in DLM21 appears to affect the *vrg* regulon as a whole. DLM21 also appears to express *vag* products normally as demonstrated by one-dimensional SDS-PAGE protein profiles, western blotting (35), and colony morphology on BGA (domed and hemolytic). Complementation of the mutation in DLM21 has not been achieved, and a specific *B. pertussis acn* mutant has yet to be constructed. Thus, the possibility that the phenotype of DLM21 is due to polar effects of the transposon on genes other than *acn* exists. However, it should be noted that aconitases have been established as regulators of gene expression (42).

Aconitases are bifunctional monomeric proteins containing an iron-sulfur cluster (4Fe-4S) which act as enzymes in the isomerization of citrate and isocitrate via cis-aconitase (42). This family of proteins can also act as translational regulators of gene expression via stabilizing or destabilizing effects on mRNA transcripts at stem-loop structures termed IREs (iron-repressive elements). Interestingly, the two functions of this family of proteins are inversely related: the enzymatic function being favored in iron-sufficient conditions, and the regulatory function being favored in conditions of low-iron or conditions such as oxidative stress which destabilize the iron-sulfur cluster. The aconitase genes can themselves be regulated by an iron-limiting response and oxidative response as seen by their Fur- and SoxRS-mediated activation in *E. coli* (24).

Although *B. pertussis* aconitase acting as indirect or direct regulator of the expression of *vrg*- or other gene products is not definitively established, the possibility of such regulation in light of the iron-limiting and oxidative environments the bacterium might encounter *in vivo* is enticing. Regulation of Bvg-regulons by iron or oxygen levels has not been described; however, *B. pertussis* contains both *fur* (7), and a *fnr*-like (6) genes which could modulate such regulation. The presence of an aconitase gene in *B. pertussis* which when mutated results in lowered expression of the *vrgs* raises the hypothesis that aconitase expression and activation, as well as *vrg* expression, may be modulated by such environmental conditions. Further work will be needed to address the validity of such hypotheses on the role of *acn* in *B. pertussis*.

A potential role for the *vrgs* in intracellular survival within eukaryotic cells has been hypothesized (33). Clearly, *vag* products have been demonstrated to be crucial for localization to an intracellular niche in epithelial cells (20, 31). We tested mutants DLM20, DLM21, and OMPR- in a HeLa cell invasion assay to address the role of the *vrgs* in invasion or survival. This seems reasonable as these mutants have reduced expression of the *vrgs* while they appear to express *vags* normally. After 7 hours invasion DLM20 and OMPR- had significantly less recoverable intracellular organisms than their parental strains. DLM21, which has higher vra expression than DLM20, showed no significant difference in the numbers of recoverable organisms. Moreover, adherence to HeLa cells was not different between the *ompR* mutants and their parental strains. Interestingly, the percentage of recoverable organisms of *ompR* mutants compared to parental strains in the invasion assay is even lower than that seen by filamentous hemagglutinin mutants (20), which presumably have lowered capacity to invade at least in part because of an adherence defect. These results raise the possibility that the *vrgs* are important in invasion or intracellular survival of *B. pertussis* in epithelial cells after initial adherence and/or invasion mediated by *vag* products. The importance of differential expression of bacterial genes in the processes of invasion and intracellular survival is well documented (18, 21). The normal capacity of DLM21 in attaining an intracellular niche could be accounted for by its residual vra expression compared to DLM20. However, it is unclear whether other genes are affected in *ompR* mutants; thus, it is possible that effects on genes other than the *vrgs* might be causing this intracellular defect.

For example, the EnvZ-OmpR regulated OmpC protein of *S. flexneri* has been shown to be important for invasion of epithelial cells (12). Hence, determining if the invasion defect seen in *ompR* mutants is due to the *vrgs* will require the analysis of mutants which are known have alterations specific to *vrg* expression.

Although a role for the Bvg-repressed products in the life of *B. pertussis* has not been clearly demonstrated, it is clear that the mechanisms that control their regulation are complex. The experiments in this chapter demonstrate a potential role for other regulatory molecules in the pathways that control the regulation of the *vrgs*. Furthermore, the ability of the *vrgs* to potentiate an intracellular niche needs to be further examined considerating the invasion defect of *B. pertussis ompR* mutants for HeLa cells. Further study on the regulation and function of *B. pertussis vrgs*, and their products, may demonstrate a role for their complex nature in this obligate human pathogen.

TABLE III.1. Strains and Plasmids

Strain or Plasmid	Relavant feature(s)	Source and/or reference
Strain		
B. pertussis		
BP338	Tohama I, Nal^r	Dr. A. Weiss (59)
BP347	BP338, *bvgS1*::Tn*5*	"
TM1081	BP338, *fhaB-lacZ vrg6-phoA*	Dr. T. Merkel (36)
TM1081-T1	TM1081, *bvgR* ::Tn*5*	"
TM1081-T16	TM1081, *bvgR* ::Tn*5*	"
TM1126	TM1081, linker insertion in *bvg R*	"
TM1210	TM1081, linker insertion in *bvg R*	"
BP370	Bvg+ Tohoma III derivative, Rif^r, Sm^r	(51)
SJ301	BP370, *bvgS-C1*	Dr. J. Milller (37)
SJ303	BP370, *bvgS-C3*	"
SJ304	BP370, *bvgS-C4*	"
DLM20	BP338, *ompR*::Tn*5*	This study
DLM21	BP338, *acn*::Tn*5*	"
BP536	BP338, Str^r	Dr. S. Stibitz (54)
OMPR-	BP536, Δ*ompR*	Dr. D. Maskell (32)
TS11a	BP536, chromosomal insertion of pT1a	This study
E. coli K-12		
DH5α	High-efficiency transformation	Bethesda Research Laboratories
XL1-Blue	High-efficiency transformation	(14)
C118λpir	conjugation proficient donor	(26)
MM294	conjugation helper strain	(5)
S17.1	Km^s mobilizing strain, IncP1 Tra functions	(46)
Plasmids		
pBSKS-	Ap^r general cloning vector	Stratagene
PBBR1MCS-4	Ap^r cloning vector, broad-host-range	Dr. K. Peterson (29)
pUT::miniTn*5*Km	Ap^{r}Km^r Transposon delivery plasmid, *oriT*	(15)
pRK2013	Km^r mobilizing plasmid, IncP1 *tra oriE1*	(17)
pSS1577	Ap^r Km^r *rpsL oriT cos*	Dr. S. Stibitz (52)
p20NotI	Km^r *NotI* clone from DLM20 in pBSKS-	This study
p20XhoI	Km^r *XhoI* clone from DLM20 in pBSKS-	"
p21ClaI	Km^r *ClaI* clone from DLM21 in pBSKS-	"
p21EcoRV	Km^r *Eco*RV clone from DLM21 in pBSKS-	"
p9.4	*Sau*3A1 clone from DLM21 in pBBRMCS-4	"
p10.1	*Sau*3A1 clone from DLM21 in pBBRMCS-4	"
pT1a	*Eco*RI fragment from p10.1 in pSS1577	"

TABLE III.2. BLAST ANALYSIS OF DLM20 Tn*5*-FLANKING REGIONS

BLAST Results Description List

BLAST Analysis for Sequence: DLM20
Search from 1 to 626 Program: blastn
Expect: 10 Observed: 0 Matrix: N/A

DataBase: Non-redundant GenBank+EMBL+DDBJ+PDB sequences
Build Date: Jan 9, 1998 8:14 AM

N	Sequences producing High-scoring Segment Pairs:		High Score	Smallest Sum Probability P(N)
1.	gi|1050720	N.meningitidis ompR gene	426	4.1e-25
2.	gi|2108341	Y.enterocolitica ompR and envZ genes	391	3.4e-22
3.	gi|2613087	Yersinia enterocolitica OmpR (ompR) gene,..	364	5.9e-20
4.	gi|2367219	Escherichia coli , pckA, envZ, ompR, greB..	347	1.5e-18
5.	gi|453286	E.coli ompB operon: ompR and envZ genes c..	347	1.5e-18
6.	gi|2613083	Shigella flexneri OmpR (ompR) gene, parti..	338	8.6e-18
7.	gi|1524387	Rhizobium meliloti chvI gene, complete cd..	336	1.3e-17
8.	gi|606010	Escherichia coli K-12 chromosomal region ..	347	7.8e-17
9.	gi|987506	Rhizobium sp. strain NGR234 chvI gene, co..	318	4.0e-16
10.	gi|602088	S.typhi ompB operon gene.	298	1.8e-14
11.	gi|304124	Agrobacterium tumefaciens (clone pNM139) ..	289	1.0e-13
12.	gi|1903395	Aeromonas jandaei response regulator prot..	287	1.5e-13
13.	gi|2636442	Bacillus subtilis complete genome (sectio..	282	3.9e-13
14.	gi|467326	B. subtilis DNA, 180 kilobase region of r..	282	3.9e-13
15.	gi|1064780	Bacillus subtilis 36kb sequence between g..	282	3.9e-13
16.	gi|587569	S.typhi ompR and envZ genes	271	3.2e-12
17.	gi|47663	Salmonella typhimurium ompR and envZ genes	271	3.2e-12

TABLE III.3. BLAST ANALYSIS OF DLM21 Tn*5*-FLANKING REGIONS

BLAST Analysis for Sequence: DLM21
Search from 1 to 812 Program: blastn
Expect: 10 Observed: 0 Matrix: N/A

DataBase: Non-redundant GenBank+EMBL+DDBJ+PDB sequences
Build Date: Jan 9, 1998 8:14 AM

N	Sequences producing High-scoring Segment Pairs:		High Score	Smallest Sum Probability P(N)
1.	gi|2661437	Xanthomonas campestris rpfA gene, complete	739	1.5e-113
2.	gi|1666697	Bradyrhizobium japonicum aconitase (acnA)..	708	6.1e-108
3.	gi|2183254	Mycobacterium avium strain GIR10 transcri..	539	3.9e-75
4.	gi|40894	E.coli acn, cysB and ribA genes	408	2.8e-30
5.	gi|1787523	Escherichia coli , yciL, btuR, yciK, sohB..	408	7.8e-30
6.	gi|1742071	E.coli genomic DNA, Kohara clone #254(28...	408	1.2e-29
7.	gi|2274852	Anopheles gambiae mRNA for putative iron ..	457	1.4e-27
8.	gi|1742089	E.coli genomic DNA, Kohara clone #255(28...	408	1.7e-23
9.	gi|1405443	B.subtilis DNA (26.2 kb fragment; 170 deg..	396	1.7e-22
10.	gi|2634090	Bacillus subtilis complete genome (sectio..	396	1.7e-22
11.	gi|599624	A.thaliana mRNA for aconitase (ZAPII)	387	9.6e-22
12.	gi|52735	M.musculus mRNA for iron responsive eleme..	328	9.1e-21
13.	gi|393206	Rattus norvegicus iron-responsive element..	346	2.5e-18
14.	gi|165029	Oryctolagus cuniculus ferritin repressor ..	172	1.2e-11
15.	gi|896472	Human iron-responsive element-binding pro..	211	1.6e-10
16.	gi|33962	H.sapiens mRNA for iron regulatory factor	211	1.7e-10
17.	gi|599623	A.thaliana Aco gene	198	5.1e-06

Figure III.1

Western immunoblot analysis of BvgS-constitutive mutants.

Numbers to the left of the panels represent the apparent molecular masses of protein standards in kilodaltons. Lanes: MW, Gibco-BRL pre-stained low molecular weight protein standards; 1, BP370; 2, BP370 modulated (m); 3, SJ301; 4, SJ301m; 5, SJ303; 6, SJ303m; 7, SJ304; 8, SJ304m. Probed simultaneously with anti-vra-a antibody (1G7-8 culture supernatant) and anti-vra-b antibody (7H1A1-5 culture supernatant).

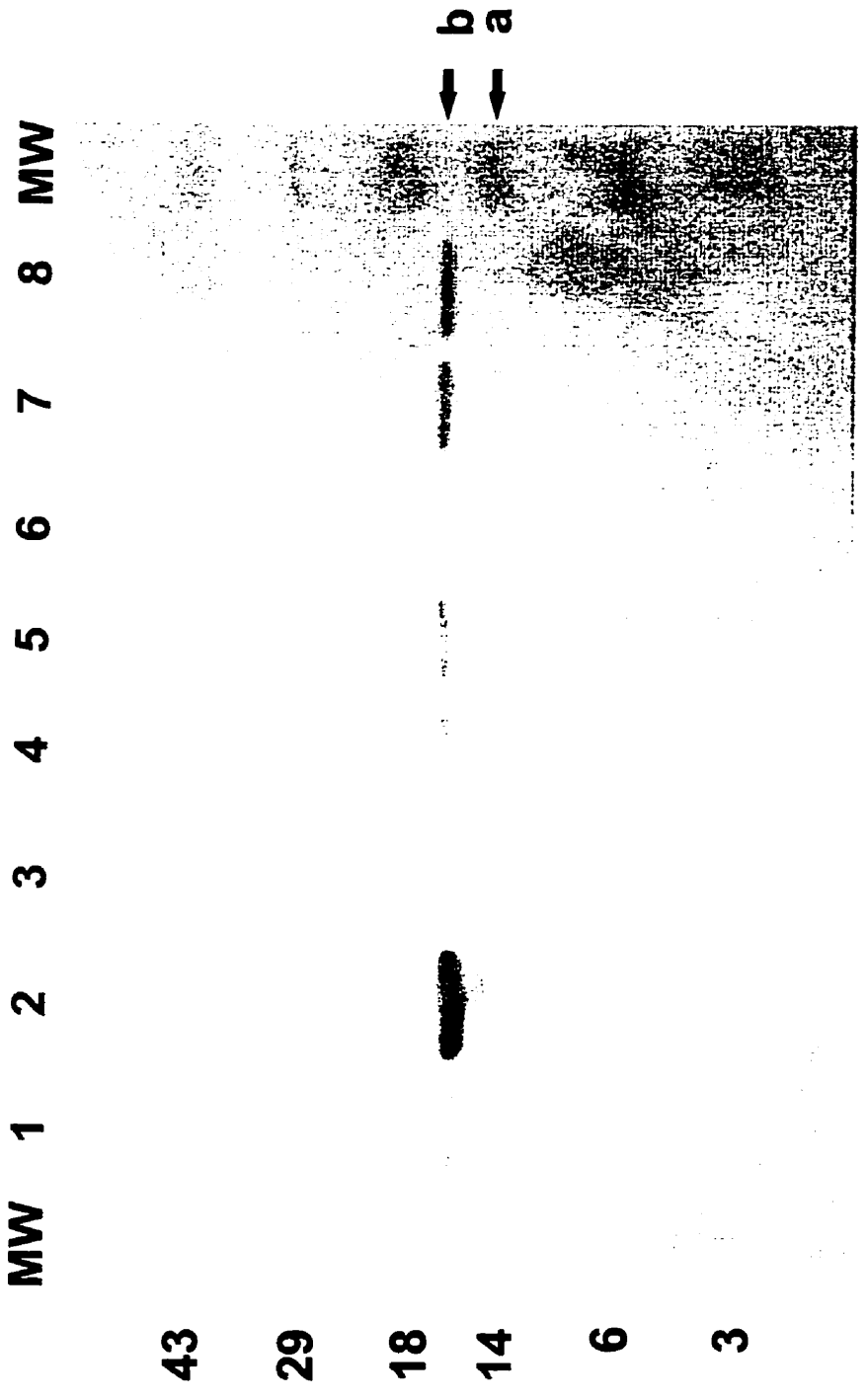

b
a
MW
8
7
6
5
4
3
2
1
MW
43
29
18
14
6
3

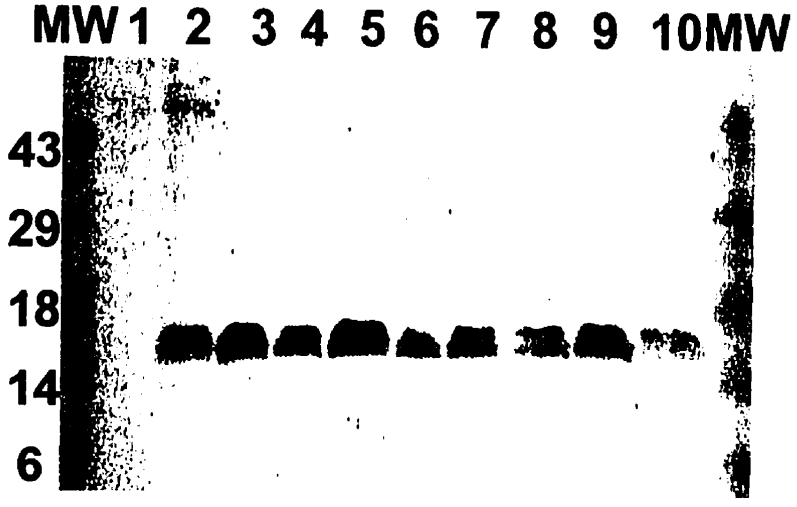
Figure III.2

Analysis of *vir*-repressed antigen expression of *bvg*R disrupted mutants by Western immunoblot.

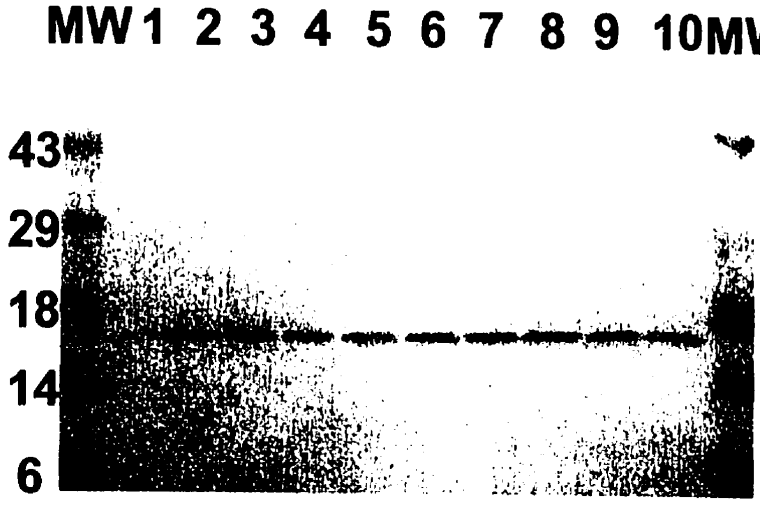
Numbers to the left of the panels represent the apparent molecular masses of protein standards in kilodaltons. Lanes: MW, Gibco-BRL pre-stained low molecular weight protein standards; 1, TM1081; 2, TM1081 modulated (m); 3, TM1081-T1; 4, TM1081-T1m; 5, TM1081-T16; 6, TM1081-T16m; 7, TM1126; 8, TM1126m; 9, TM1210; 10, TM1210m.

A. Immunoblot probed with anti-vra-a antibody (1G7 culture supernatant).

B. Immunoblot probed with anti-vra-b antibody (7H1A1 culture supernatant).



A



B

Figure III.3

Analysis of *vir*-repressed antigen expression in Tn*5* mutants DLM20 and DLM21 by Western immunoblot.

Numbers to the left of the panels represent the apparent molecular masses of protein standards in kilodaltons. Lanes: MW, Gibco-BRL pre-stained low molecular weight protein standards; 1, BP338; 2, BP338 modulated (m); 3, DLM20; 4 DLM20m; 5, DLM21; 6, DLM21m.

A. Immunoblot probed with anti-vra-a antibody (1G7 culture supernatant).

B. Immunoblot probed with anti-vra-b antibody (7H1A1 culture supernatant).

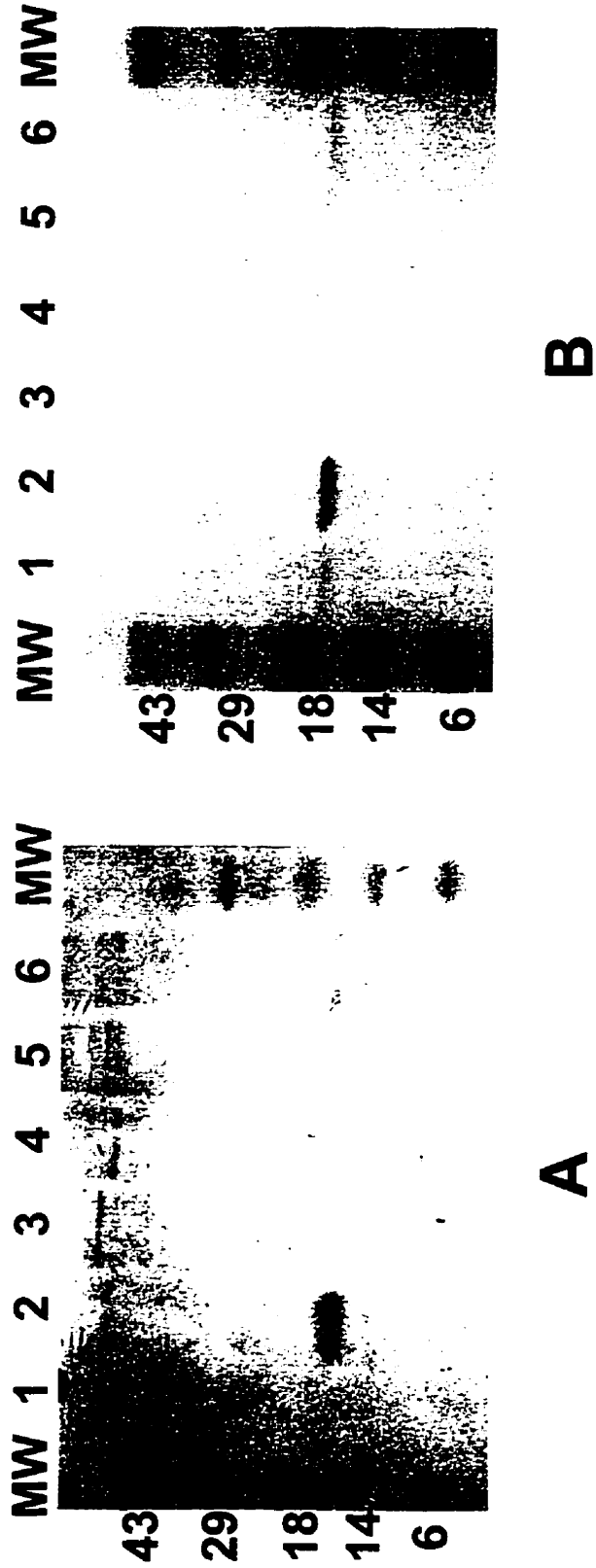

MW 1 2 3 4 5 6 MW
43
29
18
14
6
A
MW 1 2 3 4 5 6 MW
43
29
18
14
6
B

Figure III.4

Southern analysis of Tn*5* mutants DLM20 and DLM21.

Lanes: bp, numbers represent the size of Gibco-BRL 1 kb DNA Ladder standards in base pairs; a, *Apa*I; b, *Cla*I; c, *Eco*RV; d, *Sac*I; e, *Sal*I; f, *Xho*I.

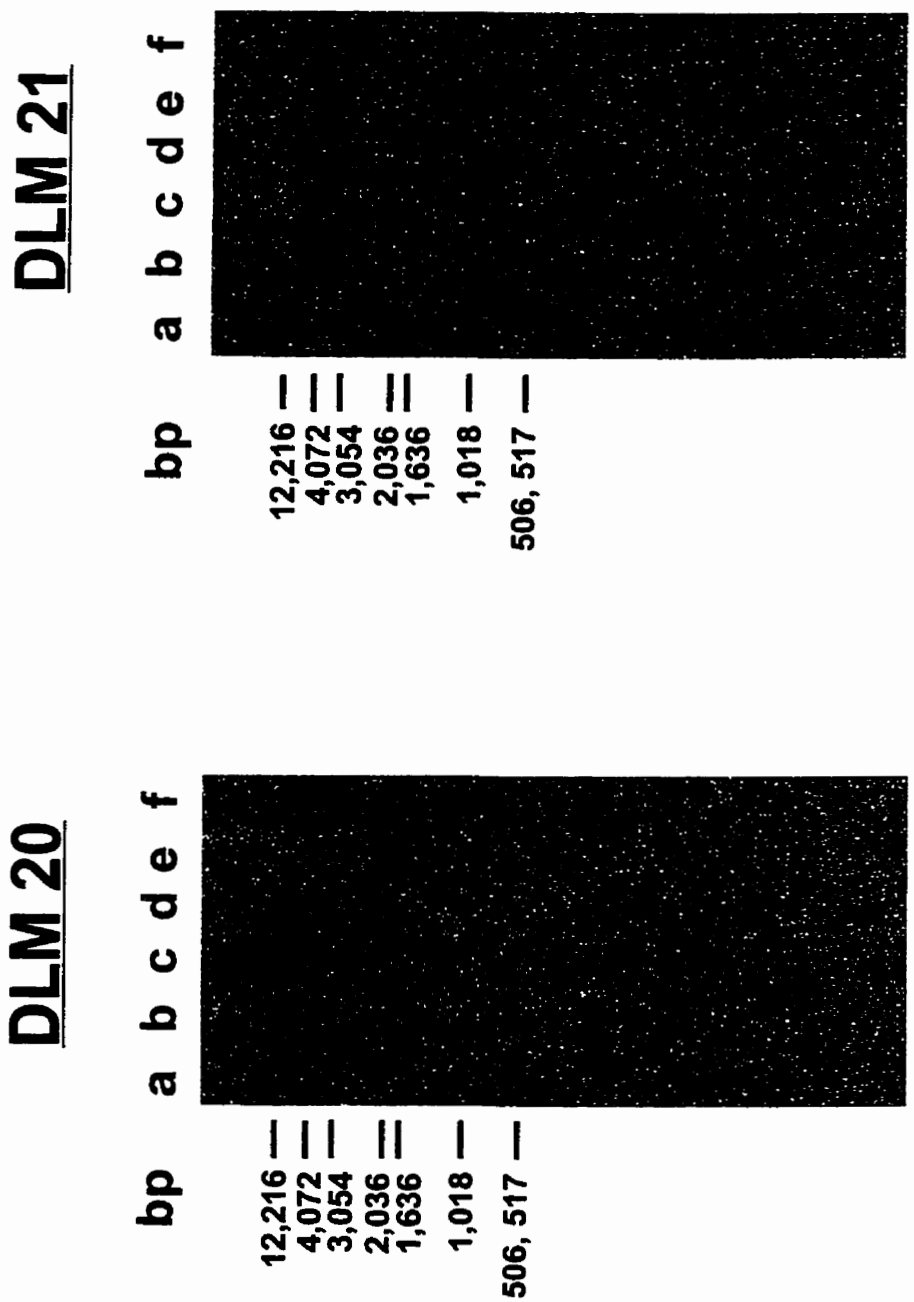

DLM 21
bp
a b c d e f
12,216
4,072
3,054
2,036
1,636
1,018
506, 517
DLM 20
bp
a b c d e f
12,216
4,072
3,054
2,036
1,636
1,018
506, 517

Figure III.5

Western immunoblot analysis of an *ompR* mutant.

Numbers to the left of the panels represent the apparent molecular masses of protein standards in kilodaltons. Lanes: MW, Gibco-BRL pre-stained low molecular weight protein standards; 1, BP536; 2, BP536 modulated (m); 3, OMPR-; 4, OMPR-m. Probed simultaneously with anti-vra-a antibody (1G7-8 culture supernatant) and anti-vra-b antibody (7H1A1-5 culture supernatant).

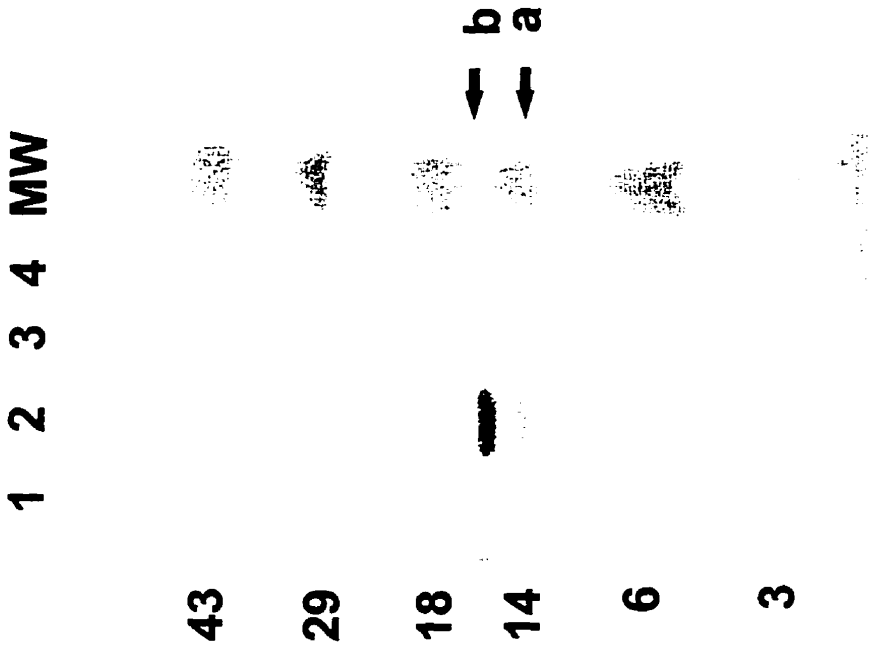

1 2 3 4 MW
b
a
43
29
18
14
6
3

Figure III.6

Western immunoblot analysis of *B. pertussis* containing pBBR1MCS derivatives.

Numbers to the left of the panels represent the apparent molecular masses of protein standards in kilodaltons. Lanes: MW, Gibco-BRL pre-stained low molecular weight protein standards; 1, BP338 (pBBR1MCS-4); 2, BP338 (pBBR1MCS-4) modulated (m); 3, BP338 (p9.4); 4, BP338 (p9.4)m; 5, BP338 (p10.1); 6, BP338 (p10.1)m; 7, DLM21 (pBBR1MCS-4); 8, DLM21 (pBBR1MCS-4)m; 9, DLM21 (p9.4); 10, DLM21 (p9.4)m; 11, DLM21 (p10.1); 12, DLM21 (p10.1)m. Probed simultaneously with anti-vra-a antibody (1G7-8 culture supernatant) and anti-vra-b antibody (7H1A1-5 culture supernatant).

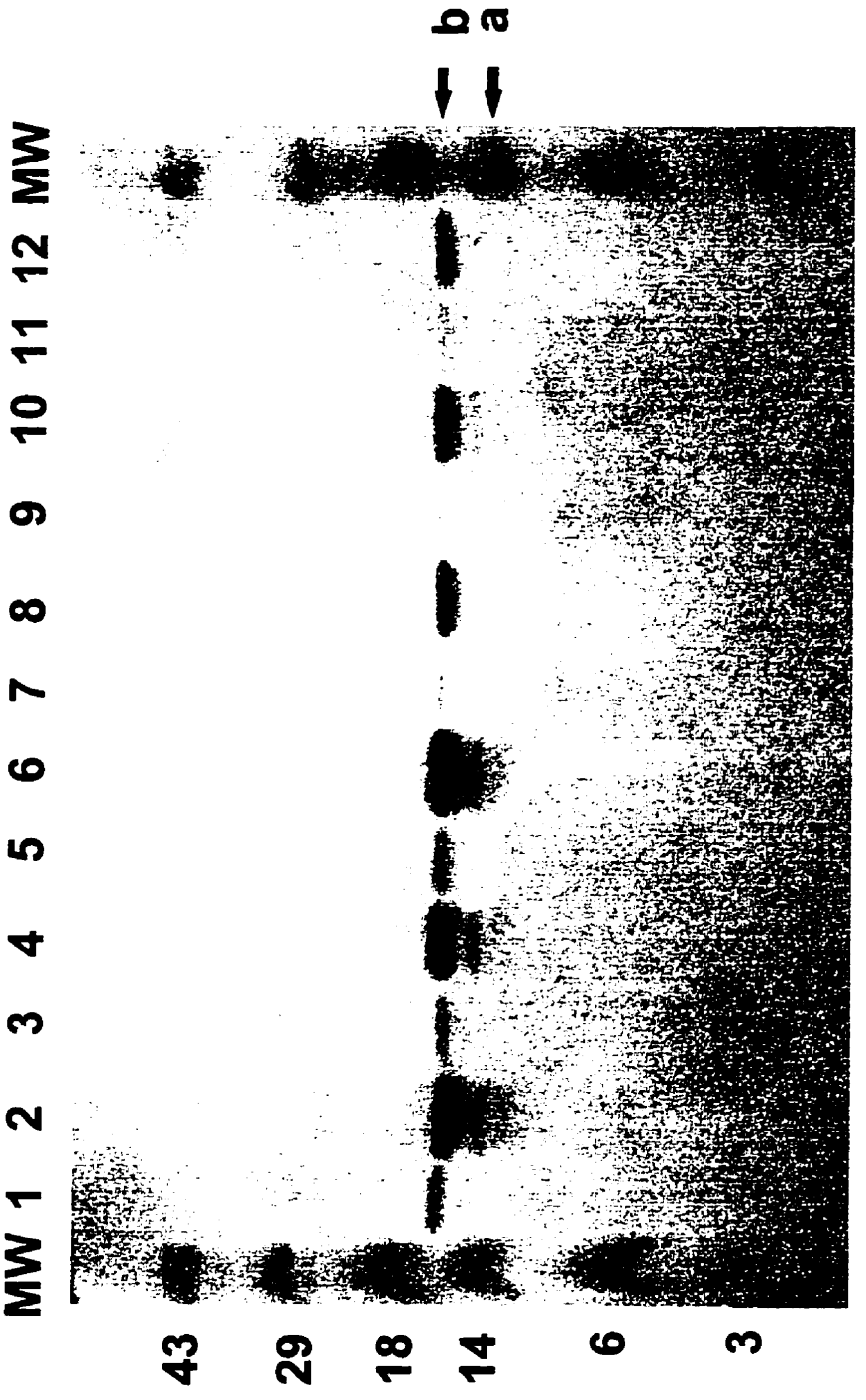

MW 1 2 3 4 5 6 7 8 9 10 11 12 MW
b
a
43
29
18
14
6
3

Figure III.7

Southern analysis of Tn*5* mutants DLM20 and DLM21 after PFGE.

Letters on the left side of the panels represent the prototype *B. pertussis Spe*I chromosomal fragment designations. Letters on the right side of the panels represent the prototype *B. pertussis Xba*I chromosomal fragment designations. Lanes: λ, New England Biolabs Lambda Ladder PFG Marker, a, BP338; b, BP347; DLM20; b, DLM21.

A. Ethidium bromide stained gel.

B. Autoradiogram of Southern of A probed with mini-Tn*5*.


SpeI
XbaI
λ a b c d λ a b c d λ
B

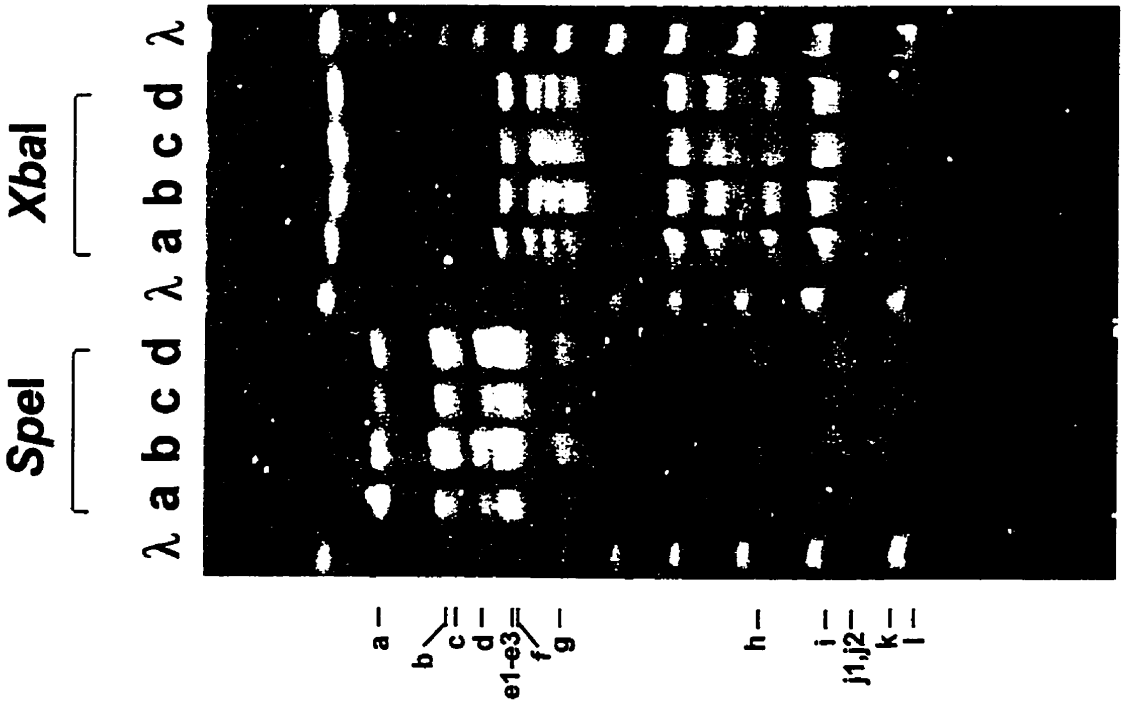

SpeI
XbaI
λ a b c d λ a b c d λ
a
b
c
d
e
f
g
h
i
k1,k2
l1-l4
m
n
o
p
q
r
a
b
c
d
e1-e3
f
g
h
i
j1,j2
k
l
A

Figure III.8

Chromosomal mapping of *acn* in strain TS11a by PFGE.

Letters on the left side of the panels represent the prototype *B. pertussis Spe*I chromosomal fragment designations. Letters on the right side of the panels represent the prototype *B. pertussis Xba*I chromosomal fragment designations. Arrows indicate the presence of bands not present in BP536. Lanes: λ, New England Biolabs Lambda Ladder PFG Marker, a, BP536; b, TS11a; c, BP536; d, TS11a.

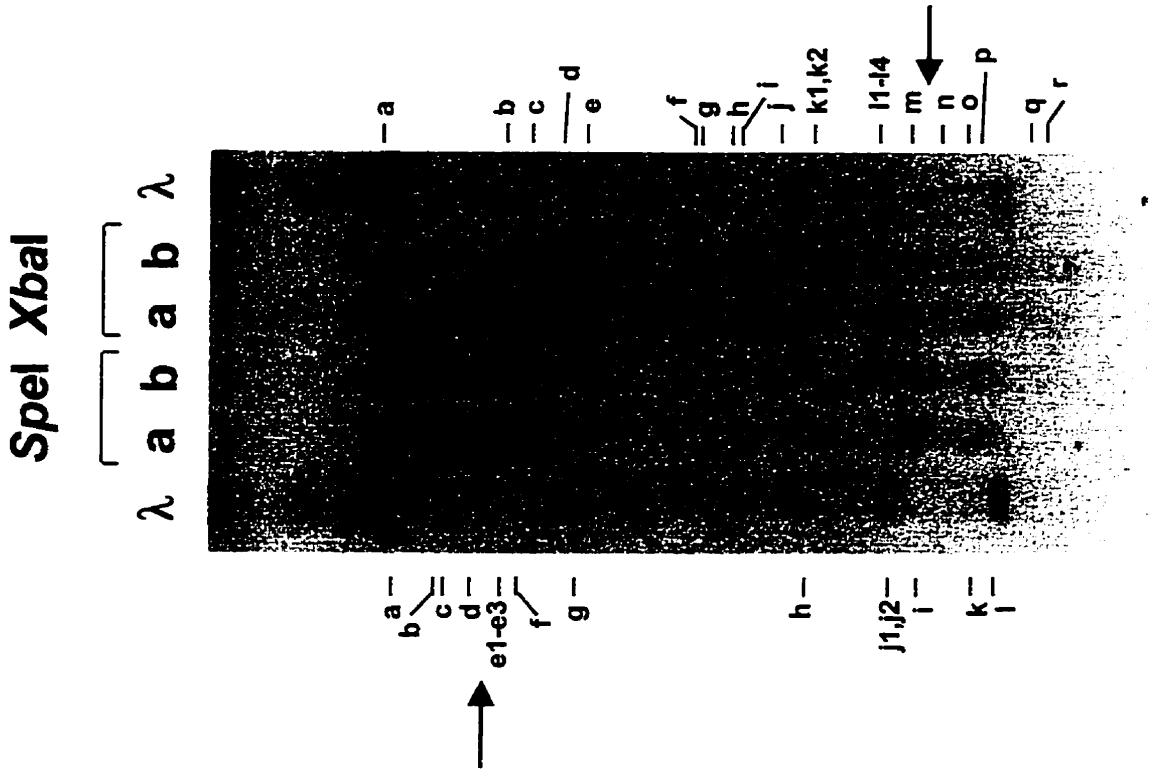
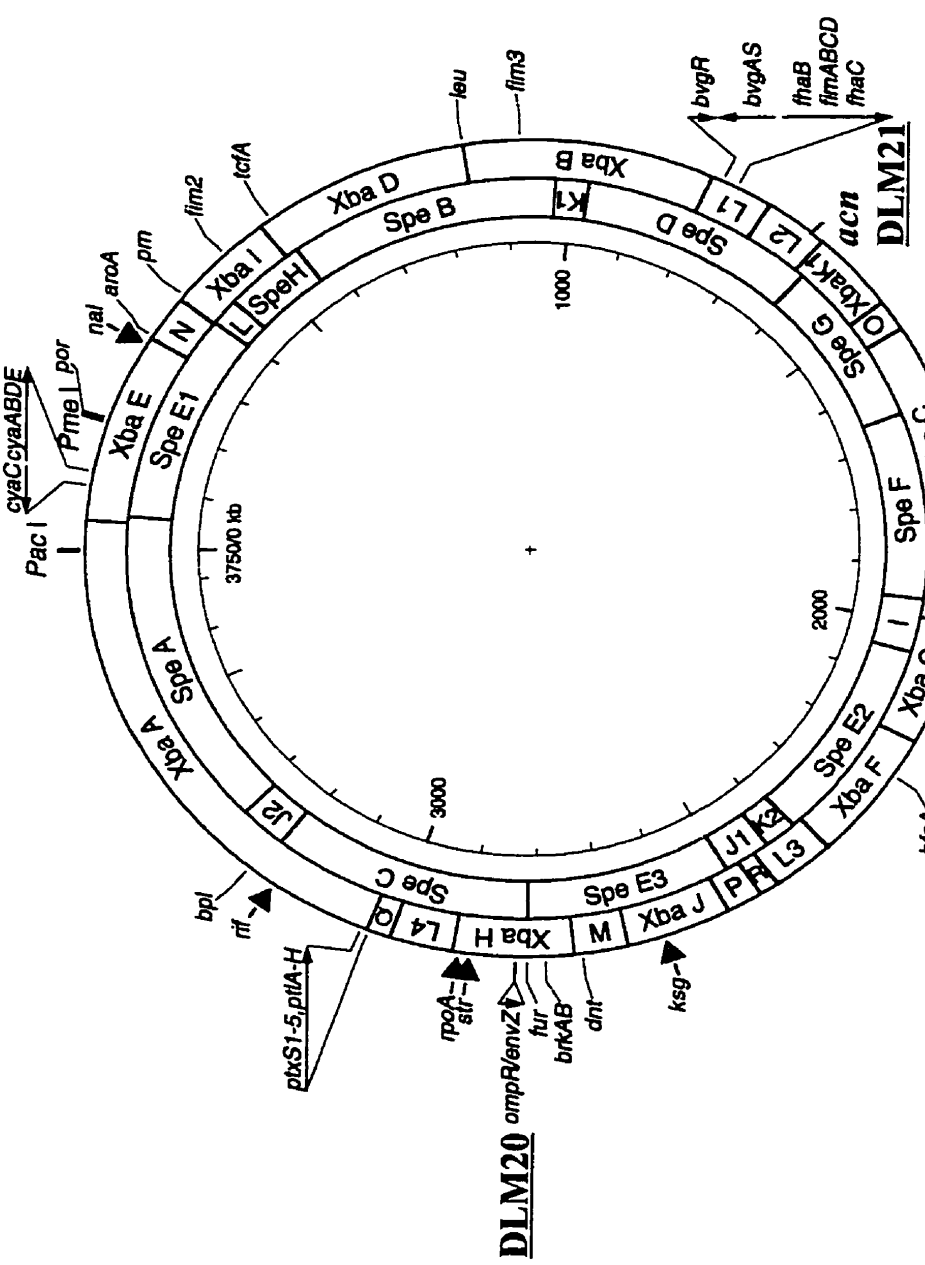

SpeI XbaI
λ a b a b λ
a
b
c
d
e
f
g
h
i
j
k1,k2
l1-l4
m
n
o
p
q
r
b
a
c
d
e1-e3
f
g
h
j1,j2
i
k
l

Figure III.9

Chromosomal locations of *ompR* and *acn* in *B. pertussis*.

The positions of Tn*5* inserts in the mutants DLM20 and DLM21 on the physical chromosomal map of *B. pertussis* are indicated by underlined text.



-map provided by Dr. Scott Stibitz

Figure III.10

Invasion of HeLa 299 monolayers by BP338 mutants.

Values represent 1000's of CFU recovered from gentamycin-treated monolayers, mean ± SEM for 4 wells per experiment replicated in 4 seperate experiments. *, $P < 0.01$, §, $P > 0.25$ compared to wild type.

Invasion of HeLa 299 Monolayers by *B. pertussis* 338 Transposon Mutants

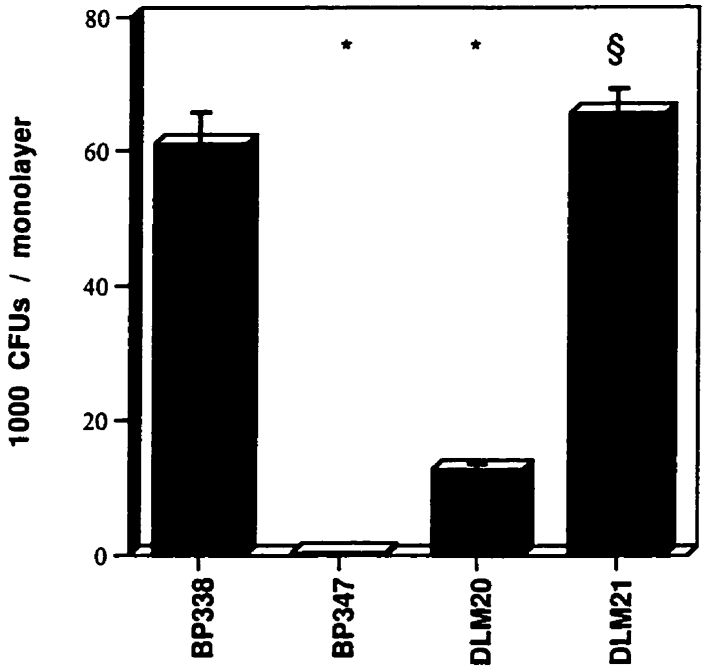


Figure III.11

Invasion of HeLa 299 monolayers by an OmpR- mutant.

Values represent 1000's of CFU recovered from gentamycin-treated monolayers, mean ± SEM for 4 wells per experiment replicated in 4 seperate experiments. *, $P < 0.01$, §, $P > 0.25$, compared to wild type.

Invasion of HeLa 299 Monolayers by *B. pertussis* 536 and OmpR- mutant

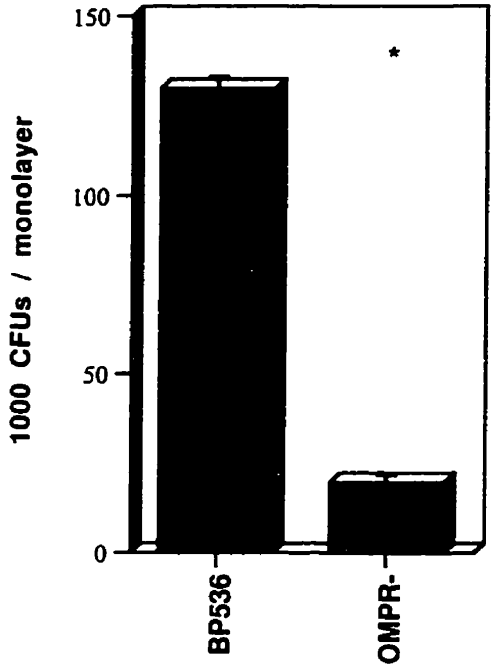


Figure III.12

Adherence to HeLa 299 monolayers by BP338 mutants.

Values represent 1000's of CFU recovered from washed monolayers, mean ± for 4 wells per experiment replicated in 5 seperate experiments. *, $P < 0.01$, §, $P > 0.25$, ◊, $P = 0.05$ - 0.25 compared to wild type.

Adherence to HeLa 299 Monolayers by *B. pertussis* 338 Transposon Mutants

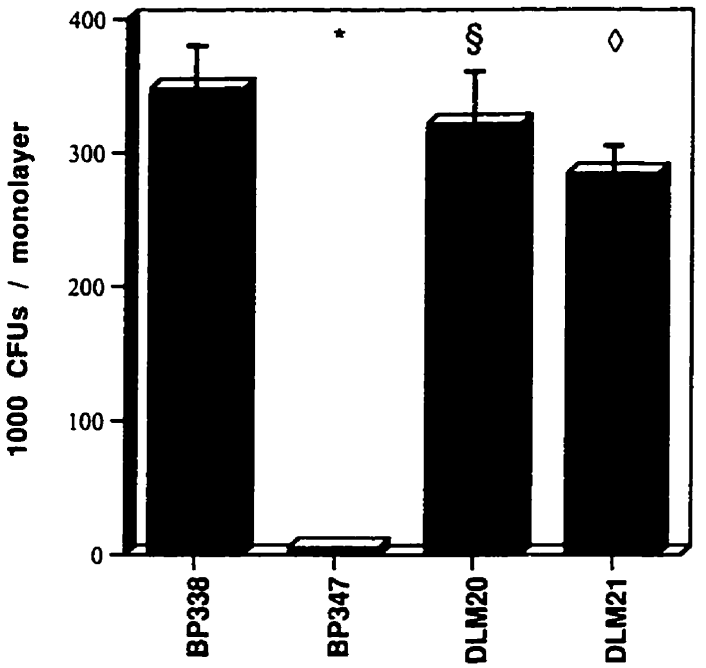
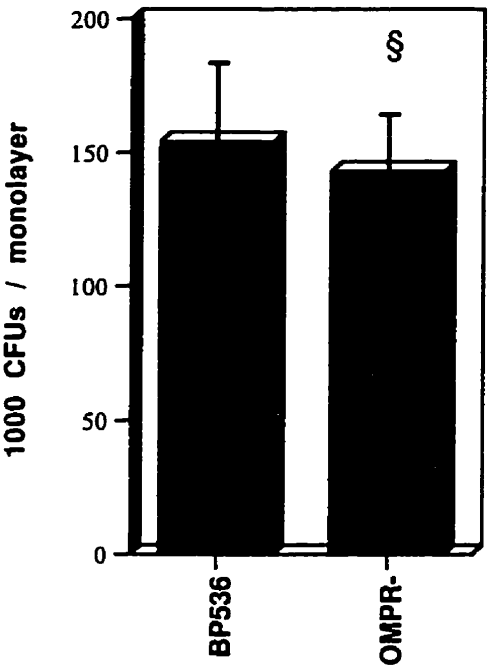


Figure III.13

Adherence to HeLa 299 monolayers by an OmpR- mutant.

Values represent 1000's of CFU recovered from washed monolayers, mean ± SEM for 4 wells per experiment replicated in 5 seperate experiments. *, $P < 0.01$, §, $P > 0.25$, compared to wild type.

Adherence to HeLa 299 Monolayers by *B. pertussis* 536 and OmpR- mutant



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Chapter IV

DISCUSSION

The findings described in this thesis contribute to the understanding of the phenomenon of phenotypic modulation via Bvg regulation in the human pathogen *Bordetella pertussis*. Moreover, they provide knowledge and tools which will assist in the further study of modulation with the goal of understanding its potential role in pathogenesis or survival of this bacterium. When our studies on modulation began, limited knowledge about the Bvg-repressed products of *B. pertussis* was available with only five Bvg-repressed genes (*vrgs*) of unknown function being described (8, 27). Thus, we sought to investigate the prevalence of Bvg-repressed proteins in *B. pertussis* with an emphasis towards discovering surface proteins which are likely candidates for molecules that interact directly with the organism's environment. Our analysis demonstrates that *B. pertussis* produces many membrane proteins (at least 22 by two-dimensional electrophoresis) specific to the modulated (C-mode) phenotype. Furthermore, a number of these proteins are present on the surface of the bacterium. To assist in the analysis of the process and products of modulation we also developed a hybridoma bank which produces monoclonal antibodies specific to C-mode *B. pertussis*. These antibodies have proved to be valuable reagents for characterizing two C-mode specific surface proteins (vra-a and vra-b). These antibodies have also aided in the search for mutants which show altered

expression of Bvg-repressed molecules. The two mutants discovered demonstrate two additional loci (*ompR* and *acn*) which are important for expression of Bvg-repressed molecules. Further study of these loci will undoubtedly expand our knowledge of the molecular mechanisms of *vrg* regulation. In addition, analysis of these and other mutants in animal and tissue culture models may greatly contribute to our understanding of the purpose of modulation. For example, we have demonstrated that *ompR* mutants, which have a defect in *vrg* expression, also have a reduced capacity to invade HeLa cells; hence, further analysis of this defect may demonstrate whether modulation is relevant to cellular invasion *in vivo*.

As is common in research, for each question we addressed; more questions were raised, and much interesting further research is suggested from our work and that of others. This chapter reviews modulation in *B. pertussis* including the findings presented in this thesis. This is done within the context of this phenomenon in the related mammalian pathogen *B. bronchiseptica*, and within the context of regulation of pathogenic mechanisms of other bacteria. In addition, further experimentation and areas of research which may clarify the mechanisms and role of modulation are explored.

A. WHY MODULATE?

Over the last 25 years, knowledge about the virulence factors and pathogenic mechanisms of *Bordetella pertussis* has expanded greatly. The molecular genetic and biochemical analysis of the *B. pertussis* virulence factors has enhanced our knowledge of their complexity in terms of their structures, functions, and roles in pathogenesis (58). Likewise, it has become increasingly evident that their regulation, and the machinery that controls it, are also very elaborate (52, 53). The complex BvgAS two-component regulatory system is paramount in the coordinate regulation of virulence factors in *B. pertussis*. Most of the major virulence factors are positively regulated by Bvg, and sensing of specific environmental factors by it results in the reduction of expression of these Bvg-activated factors and a switching of the phenotype of the bacterium from X-mode to C-mode.

The ability of bacteria to coordinately regulate the expression of sets of genes in response to environmental stimuli allows pathogens to express gene products when they are appropriate to an environmental niche. These switches in gene expression are important for such processes as colonization, pathogenesis, evasion of a host-immune response, survival in environments outside the host, transmission, and persistence in host micro-environments (17, 19). Timely gene expression also conserves metabolic energy in contrast to constitutive production of these products.

In *B. pertussis*, a role for modulation has been proposed by consideration

of the differential expression of Bvg-activated factors (49). Some of these factors, such as the adhesin filamentous hemagglutinin, are expressed early in the activation process; while expression of other factors, such as adenylate cyclase toxin and pertussis toxin, occurs later. This regulation has been reasonably hypothesized to have evolved due to the need for the bacterium to express attachment factors early in the infection process, and to express toxins later when they are required for pathogenesis and evasion of host immune responses. In this model, *B. pertussis* would be in C-mode prior to inhalation by the host, as ambient temperature is one of the modulating signals sensed by Bvg (38). However, it is unclear whether the bacterium would encounter these low temperatures long enough to modulate as *B. pertussis* is believed to be transmitted person to person by aerosol droplets. Furthermore, this model does not speculate on the possibility of a specific role for the C-mode of the bacterium within the host.

The seminal work of Lacey (28) suggested through antigenic analysis that modulation to the C-mode resulted in the production of C-mode specific proteins and not just the loss of X-mode proteins. However, due to the obvious complexity and importance of the Bvg-activated X-mode specific factors, it may have seemed that the avirulent C-mode bacteria are a dormant phenotype, with the lack of these factors and a comparatively unadorned surface composed of molecules common to both modes. Work from the laboratory of Dr. John Mekalanos (27) demonstrated the existence of the Bvg-repressed genes (*vrgs*) which encode unique proteins of the C-mode. These genes were discovered

through the detection of PhoA expression of Tn*phoA* insertion mutants, suggesting that the mutated genes encode proteins which are exported at least to the periplasm of the bacterium as this is required for alkaline phosphatase activity (31). To search for Bvg-repressed surface proteins of *B. pertussis* we analyzed outer-membrane vesicles by two-dimensional protein electrophoresis and quantitative staining with Coomassie blue. What is immediately noticeable from this analysis is that C-mode cells produce many membrane proteins that are increased relative to X-mode cells (at least 22). Some of these molecules are surface exposed by radiolabeling techniques, while at least two have been definitively shown to be surface exposed by immunological techniques. Hence, it is incorrect to think of phenotypic modulation a just a switch from a virulent to a quiescent state as it is clear that the bacterium not only invests metabolic energy and coding sequence for the ability to switch off the major virulence factors, but it also invests energy and genome for the ability to express a whole set of membrane-associated Bvg-repressed factors when modulated.

However, discovering a role for modulation with respect to these Bvg-repressed genes and products has remained elusive. One investigation has proposed a role for modulation *in vivo* after discovering that a transposon mutant had decreased virulence in a mouse model of infection, and lessened ability to colonize the mouse trachea and lungs (8). However, these conclusions have been recently disputed as a precise mutation of the disrupted locus did not result in the same phenotype; thus the original defect was likely due to polar effects caused by the transposon (34). Furthermore, the authors found no evidence for

modulation of *B. pertussis* in the mouse model, and showed that constitutive expression of the *vrgs* decreased colonization. However, it is questionable whether this is an appropriate model in which to study subtle changes in virulence or pathogenesis that might occur as a result of an alteration in expression of the C-mode phenotype. In another study, modulation was proposed to occur based on the decreased production of a Bvg-activated factor, adenylate cyclase, upon entry of human monocytes (36). However, the production of adenylate cyclase was measured as a function of enzyme activity of protein isolated from intracellular bacteria, and the difference was a merely three-fold difference during the time course of infection while intracellular bacterial viability decreased at least two logs. Moreover, the conclusions of a subsequent study by the same author contradicted the original report demonstrating that cAMP and adenylate cyclase toxin production within monocytes increased survival (37).

Studies of the relevance of modulation in the veterinary pathogen *B. bronchiseptica* have provided far more information, and furthermore can be done in the relevant host. In *B. bronchiseptica*, the X-mode phenotype has been shown to be sufficient for infection and virulence in animal models (1, 13, 14). Furthermore, no immunological evidence for modulation was found, and ectopic expression of a Bvg-repressed factor, flagella, was seen to impede infection.

It remains to be seen what conclusions can be drawn from these studies with respect to the infection *of B. pertussis* in humans. Immunological evidence for modulation of *B. pertussis* in humans has not been found, but the C-mode

antigens of *B. pertussis* is noted to be poorly immunogenic (chapter II; refs. 8, 28), and if modulation does occur it may be in a site which is immunologically privileged. In addition, *B. bronchiseptica*, although closely related to *B. pertussis*, has many significant physiological differences. It is known to be much less nutritionally fastidious, and has the ability to survive in environments outside its mammalian hosts (15, 44, 45). In contrast, *B. pertussis* is nutritionally fastidious and has not been described to have an reservoir other than humans. Interestingly, while the Bvg-activated factors of the two organisms are almost completely shared, the Bvg-repressed factors appear unique between the two species. In *B. bronchiseptica*, production of siderophore and flagella are Bvg-repressed phenotypes (2, 3, 22), and production of these phenotypes is speculated to be important in survival or transmission of the organisms in environmental niches outside its hosts (14). *B. pertussis* is non-motile (although it contains sequences homologous to flagellar genes), and production of siderophore is not a Bvg-repressed trait. Studies, by Beattie *et al.* have shown that *B. pertussis* expresses the Bvg-repressed gene *vrg-6*, while related *Bordetellae* do not, though they appear to contain a silent copy of the gene (8). Similarly, in chapter II we have shown that Bvg-repressed vra-a and vra-b expression is unique to *B. pertussis*. Hence, it is reasonable to speculate that the Bvg-repressed factors of the two species have evolved to fulfill the different needs encountered by the two bacteria in their respective ecological niches.

The mechanisms by which *B. pertussis* and *B. bronchiseptica* regulate their Bvg-repressed products also differ. Regulation of the Bvg-repressed

motility in *B. bronchiseptica* is controlled directly though transcriptional repression by BvgA at the *frl* and *fla* loci (2, 3). In contrast, *B. pertussis vrg* repression is controlled by the production of Bvg-activated BvgR which acts as a transcriptional repressor (6, 7, 39). In Chapter III, we demonstrated that vra-a and vra-b are subject to repression by BvgR, analogous to the repression of the *vrgs* by BvgR. In addition, we have described two putative regulatory loci, *acn*, and *ompR*, which are required for the full expression of Bvg-repressed molecules. It will be interesting to determine if these loci regulate gene expression in other *Bordetella* species.

The Bvg-intermediate mode of *B. bronchiseptica* has recently been demonstrated (15), and is likely analogous to the I-mode *of B. pertussis* originally described by Lacey (28). Preliminary investigations demonstrate vra-b to act as an I-mode protein in at least one *B. pertussis* strain (32). Further investigations may demonstrate an I-mode specific phenotype of *B. pertussis* which produces a number of I-phase specific molecules which have a role in the life-cycle of this pathogen.

The sensitivity of *B. bronchiseptica* strains to *in vitro* modulators has been demonstrated to be greater than the sensitivity of most *B. pertussis* strains (33). This increased sensitivity has been shown to be due to specific amino acid differences between the species near the primary site of phosphorylation in BvgS. It has been speculated that these differences may be due to a selective pressure to maintain the ability to modulate in *B. bronchiseptica* that is not present in *B. pertussis*; implying that modulation is not significant for

B. pertussis. The significance of this moderate increase in sensitivity (about 4-fold) is questionable considering that the *in vitro* modulators are not believed to be relevant in the environments *of B. pertussis* at these concentrations. Moreover, if lack of selective pressure results in random mutational inactivation of the Bvg repressed regulon, one would expect to see an effect across the Bvg-repressed molecules and regulatory factors. However, this is not seen as *B. pertussis* not only has maintained a intact Bvg-repressed regulon, but one which appears unique in composition and regulation to that of *B. bronchiseptica*. For example both *vrg-6*, and vra-b are Bvg-repressed specifically in *B. pertussis*, although *vrg-6* sequences are present in *B. bronchiseptica*, and it also produces a low level of vra-b which is not Bvg-regulated (chapter II; ref. 8). In contrast copies of the Bvg-activated pertussis toxin loci are present in species other than *B. pertussis*, but they are apparently silent due to an accumulation of mutations in their promoter regions (4). Thus, much more scientific investigation is warranted to determine whether modulation is significant for *B. pertussis*.

In Chapter III, we demonstrated that *ompR* mutants have a decreased ability to invade and survive within epithelial-like cells relative to wild type. In addition, we have demonstrated that *B. pertussis ompR* mutants have a greatly impaired ability to produce the vras and *vrgs*. This implies (but does not definitively demonstrate) that the Bvg-repressed regulon is required for *B. pertussis* invasion of epithelial cells. Previous investigations have shown that Bvg-activated factors are crucial for cellular invasion (18, 29)

The expression of different sets of genes at different times enabling attachment, invasion, and survival within eukaryotic cells of several pathogens is well documented (17, 19). In the intestinal pathogen, *Salmonella typhimurium*, the two-component regulatory system PhoP-PhoQ regulates virulence (24, 40). Moreover, Pho-activated genes (*pags*) and Pho-repressed genes (*prgs*) are regulated differentially to affect the entry and survival into epithelial and macrophage cell types, with *pag* loci being important in intracellular survival (20, 24). The OmpR-EnvZ system has also been shown to be required for virulence, and proper intracellular trafficking of *S. typhimurium* (24, 42). In enteropathogenic *E. coli*, initial attachment to epithelial cells results in production of proteins which induce intimate attachment to, and effacement of the cellular layer at the site of bacterial adherence (16, 46). This phenotype is triggered as a result of signals sensed by the bacterium that are produced by the eukaryotic cells after initial attachment.

Thus, the regulation of sets of pathogenic factors in the survival of microorganisms is seen to be crucial for the virulence and survival of disease causing bacteria (17, 19). However, the determination of whether *B. pertussis* modulation plays a role in its life-cycle important for establishment, pathogenesis, carriage, or transmission requires much further research. Although such a role may be difficult to demonstrate, it may be important in our understanding of the survival mechanisms of this pathogen, and the results may change the way we approach the treatment and prevention of whooping cough.

B. MODULATION: FUTURE PERSPECTIVES

1. Searching for modulation in model systems

If *in vivo* modulation occurs by *B. pertussis*, it may be hard to demonstrate due to the location and numbers of modulated bacteria within the host. For example, our lab and others have speculated that *B. pertussis* may modulate within some eukaryotic cell type to affect evasion of the immune response and facilitate persistence and carriage of the organism. Where and when this might occur is a major consideration in any experiment which might seek to demonstrate modulation. Searching for modulation *in vivo* has the limitation that it can not be readily done it's natural host: humans. *In vivo* animal models and *in vitro* tissue culture models must always be used with the concern of whether the model used is relevant to the *in vivo* situation, but they of course often provide a wealth of information that can be used to answer relevant scientific queries.

Our laboratory is interested in using the anti-vra antibodies in tissue culture models such as the HeLa cell invasion assay to look for evidence of modulation by immunofluorescence microscopy. If evidence is found, then these techniques can be tried in other *in vitro* models, and eventually in animal models and perhaps patient samples. Another methodology our laboratory is pursuing is the use of green fluorescent protein (GFP) reporters for the detection of *vrg* expression. This technique has been used successfully to monitor changes in gene expression of *S. typhimurium* within macrophages (56). The FACS-enhanced GFP now available has a high signal output (12) which can be further

enhanced by the use of anti-GFP antibodies in fluorescence experiments. When reporters are fused to strong promoters the fluorescence can effectively be used to FACS sort bacteria from model systems (55-57) to isolate clones with induced fluorescence. One of the advantages of GFP is its stability, which allows measurement of its accumulation in experiments relative to appropriate controls. However, this stability means it is not as useful for monitoring decreases in expression in time course experiments. GFP also has the advantage of being stable to fixation and is intrinsically fluorescent, and thus it does not require the addition of substrates for its detection. A plasmid *vrg-6::gfp* reporter which can be maintained in *B. pertussis* has been constructed in our lab by Gina Broitman and preliminary assessments of its utility look promising. Some of the technical difficulties that need to be addressed with use of this system for *B. pertussis* modulation is the small size of the bacterium, its slow growth rate, the relatively weak promoter strength of the *vrgs*, and the possibility that *B. pertussis* in a modulating niche may not be actively replicating.

Other techniques used to study regulation *in vivo* or in tissue culture models include the IVET (In vivo expression technology) system (30), the construction of subtractive RNA libraries (43, 54), the use of sequence-specific tags to identify transposon mutants with decreased virulence (26), and analysis of protein expression by *in situ* radiolabeling (9, 10). There are several caveats to the potential use of any of these techniques to search for modulation of *B. pertussis*. We believe that if modulation does occur the numbers of modulated bacteria may be small compared to the unmodulated bacteria on the outside of

cells, or elsewhere in animal and *in vitro* models. Thus, problems of background must be addressed in any experimental model. Furthermore, the *vrgs* may be "leaky" producing significant amounts of mRNA transcript even in the absence of derepression. Thus, they might be selected against in any procedure (such as subtractive hybridization) which seeks to clone genes specifically produced in any particular environmental niche.

The original IVET system relies on the expression of an gene essential for virulence *in vivo* to identify promoter fusions which activate a promoterless copy of the essential gene (30). Surviving organisms which pass thought the model are then tested for *in vitro* expression of the gene by means of *lacZY* reporters which are also generated in this experimental system. Bacteria which do not express the fusion *in vitro* thus help identify *in vivo* specific genes. Many of the genes cloned in this manner have turned out to have metabolic and housekeeping functions underlying the importance of these systems for full virulence *in vivo*. However, because the essential gene must always be promoted to ensure survival of the bacterium, this method can not be used to select for genes which are expressed only at particular times during infection, and thus is not a useful to tool to search for possible modulation of *B. pertussis*.

Several variants of the IVET system now exist (11). One of these which may be of particular use in *B. pertussis* produces positive selection for gene expression by removal of DNA encoding sucrose sensitivity contained between recombinase-specific sites. Expression of the promoterless recombinase gene after generation of a gene fusion results in the removal of the sensitivity locus,

and thus genes that are expressed within a model system can be selected for positively. It is not essential for the recombinase to be expressed all the time, but rather only to a certain level at one time during infection such that antibiotic resistance is generated. Hence, genes which are expressed at certain times of infection can be isolated. Modulation of *B. pertussis* could be assayed using such a system by creating a *vrg* promoter-recombinase fusion. A strain harboring this reporter could be tested on solid media to demonstrate acquisition of resistance on modulating media but not on non-modulating media as a control for the sensitivity and specificity of the system. After resistance is shown to be an accurate score for modulation, the strain could be tested *in vitro* and *in vivo*. The amount of resistant strains recovered from an experimental model compared to the amount of sensitive strains recovered would demonstrate the percentage of strains which have modulated. In addition, testing strains from different locations of such a model may help determine where modulation occurs.

Mutagenesis with transposons containing sequence-specific tags allows determination and recovery of mutants which do not survive in a model system relative to growth in normal media (26). Using such a system in a mouse model to recover mutants with decreased virulence would not likely demonstrate mutants in *vrg* loci as such models are quite stringent and even some *vag* mutants which are mutated in loci thought to be important for virulence in humans are not less virulent in these models (23). Furthermore, mutations in single *vrg* loci may not show reduced virulence even if they are involved in survival as separate *vrg* loci may have redundant functions. If expression of the

vrgs is important for virulence it may be possible to isolate transposon mutants which affect the entire *vrg* regulon (such as *ompR* mutants). However, one would expect that *vag* mutants would be isolated preferentially due to their crucial role in virulence; thus background would be a problem when searching for mutations in loci other than those of the *vag regulon*.

In chapter II, we demonstrated a Bvg-repressed specific protein profile by two dimensional electrophoresis. *In situ* protein labeling of bacterial proteins in tissue culture models have been used successfully to demonstrate changes in protein expression by two-dimensional electrophoresis when bacteria occupy a particular cellular localization (9, 10). Use of these methods to detect changes in protein expression consistent with modulation may be difficult due to the slow replicative rate of *B. pertussis*. For example, if *B. pertussis* modulates inside epithelial cells, these methods still may not be able to demonstrate it as the numbers of intracellular *B. pertussis* are relatively low and the bacteria do not appear to actively replicate (18). Similarly, problems of sensitivity may also be encountered when using anti-vra MAbs to demonstrate modulation of *B. pertussis* by protein electrophoresis of material isolated from a tissue culture system.

The use of regulatory mutants to determine the importance of modulation may also be informative. In chapter III, we demonstrated that *ompR* mutants have a decreased ability to survive in the HeLa cell invasion model. Use of these and other mutants in model systems may demonstrate an important role for the *vrgs*. It is not definitively established that the *ompR* mutants are defective only in

the expression of the *vrgs*. Thus, the analysis of other mutants such as BvgS constitutive mutants which cannot modulate, and BvgR constitutive mutants which would never derepress *the vrgs*, will be helpful in searching for a role for the Bvg-repressed regulon.

The techniques used in this thesis to demonstrate Bvg-repressed proteins, combined with the use of the C-mode specific antibodies we developed, might be used to demonstrate a *in vivo* immune response to C-mode antigens. Using pre- and post-infection antisera to probe two dimensional electrophoretic separations of *B. pertussis* protein profiles may be able to demonstrate an immune response to either vra-a or vra-b. If recombinant antigens or synthetic epitope analogues were available it would also enable us to screen antisera for reactivity against these antigens by ELISA. Cellular immune responses to *B. pertussis* are being increasingly investigated. There is data suggesting that a TH-1 immune response is required for effective immunity to *B. pertussis* (41, 48). Thus, it will be interesting to see if Bvg-repressed antigens elicit this response through testing of isolated proteins and mutants in experimental models.

Finally, determination of gene sequences to discover homologies with proteins of known function may help explain the function of the Bvg-repressed regulon. The *vrgs* currently described do not show significant homology to proteins of known function (8). Thus cloning of more *vrgs*, including those which presumably encode vra-a and vra-b, may be extremely informative. Cloning of the vras may be facilitated by use of the anti-vra MAbs. For example, protein sequence obtained from immunoprecipitated vras may be used to design

oligonucleotides which can be used to search for the vra encoding genes. Alternatively, use of the antibodies to screen expression libraries of the *B. pertussis* genome may expedite cloning of the structural vra genes. In addition, further screening for *vrgs* should be undertaken. In our lab a promoter-trap strategy using a plasmid-GFP reporter has been used with success to clone putative *vrgs*. The cloning and sequencing of these and other *vrgs* may provide valuable information regarding the function of the *vrgs* and modulation in *B. pertussis*.

2. Regulation of the Bvg-repressed regulon

Several interesting questions are raised by the findings of this thesis with respect to the molecular mechanisms of Bvg global regulation in *B. pertussis*. We have demonstrated that an intact *ompR* locus is required for expression of the *vrgs* and vras. However, we have not determined what environmental signals might be at work if OmpR is acting as a regulator of the *vrgs*. This is a particularly interesting question considering that the normal cognate activator EnvZ is apparently not produced in *B. pertussis* (35). Analysis of additional genetic constructs, and biochemical analysis of isolated regulator proteins, may help determine the hierarchy of regulation of the *vrgs* and determine a potential modulator of OmpR activity. For instance, it will be important to determine if BvgS is able to phosphorylate OmpR, and to determine if OmpR-mediated *vrg* expression acts at a point before, after, or concurrent to the repression of *vrgs* by BvgR. If OmpR acts directly as a transcriptional regulator at promoters of *vrgs*

and other genes then it will be important to describe the *cis*-acting sequences which are required for regulation. In addition, determining the relevant signals which activate OmpR and BvgS may facilitate a better understanding of the molecular pathogenic mechanisms of *B. pertussis*. For example, recent analysis of the PhoP-PhoQ regulation of *Salmonella* virulence has demonstrated that magnesium cation concentration is the signal which modulates gene expression pertinent to extra- or intracellular localization (21). Moreover, PhoP-PhoQ has been shown to interact with other regulatory systems in the sensing of multiple environmental signals (50). Interestingly, mutations which differentiate the *B. pertussis* phenotypic modulation response to specific environmental modulators have been described (51). Our lab is interested in determining whether osmolarity or other conditions are important in the expression of the Bvg-repressed regulon. Further knowledge concerning the signals and molecular mechanisms which modulate OmpR- and BvgAS-mediated *vrg* expression may help elucidate the role of modulation in whooping cough.

In Chapter III, we demonstrated the importance of the putative *B. pertussis acn* locus in full expression of the *vrgs*. Aconitases are known to be important in gene regulation and are subject to regulation and activation by conditions of low iron and oxidative stress (47). The potential importance of these conditions in expression of the Bvg-repressed regulon remains to be determined. Thus, it will be interesting to determine if *vrg* expression is sensitive to such environmental conditions. For instance, aconitase genes have Fur binding sites in their 5' regions which function in transcriptional activation (25).

The *fur* gene of *B. pertussis* has been cloned (5), and it should be determined whether mutation of this gene results in lowered *vrg* expression due to a decrease in aconitase expression. The determination of the precise molecular regulatory mechanisms of the Bvg-repressed regulon may also be relevant in determining the mechanisms of I-mode specific expression. Moreover, analysis of regulation may be crucial in understanding the function of phenotype switching in the *B. pertussis* life-cycle.

D. CONCLUSIONS

Investigations of the molecular biology of *Bordetella pertussis* pathogenesis has provided many insights into the complex strategies it employs to remain a successful human pathogen. The complex BvgAS regulatory system is paramount in the expression and regulation of the Bvg-activated regulon encoding the major virulence factors of *B. pertussis*. However, it is becoming increasing clear that the Bvg-repressed regulon is also complex in terms of its size, and the molecular mechanisms of its regulated expression. Future research into this complexity will may expand our knowledge of the survival strategies of this pathogen by providing an understanding of the role of modulation in its life-cycle. Moreover, this knowledge may have significant implications for the strategies which humans use to control the prevalence of the insidious disease whooping cough.

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APPENDIX

ADDITIONAL FIGURES

Chapter I is presented in paper format, and thus according to the guidelines of the Faculty of Graduate Studies and Research, it cannot be drastically altered from the published version. Some data not shown in chapter I are presented here as figures for completeness and review. The materials and methods, results, and discussions relevant to these figures are included in Chapter I, and these additional figures are referenced there to this appendix.

Figure A.1

Comparison of protein profiles of virulent (phase-I) *B. pertussis*, and avirulent (phase-III-like) *B. pertussis.* III.

Coomassie stained 7.5% SDS-PAGE. Numbers on the vertical axis represent the apparent molecular weights of protein standards in kDa. MW, Bio-Rad protein standards; 1, virulent phase BP338 whole cell lysate; 2, avirulent phase BP347 whole cell lysate; 3a, 15 μg BP338 OMVs; 3b, 30 μg BP338 OMVs; 4a, 15 μg BP347 OMVs; 4b, 30 μg BP347 OMVs. Arrows indicate positions of bands which are increased in avirulent phase OMVs relative to virulent phase OMVs.

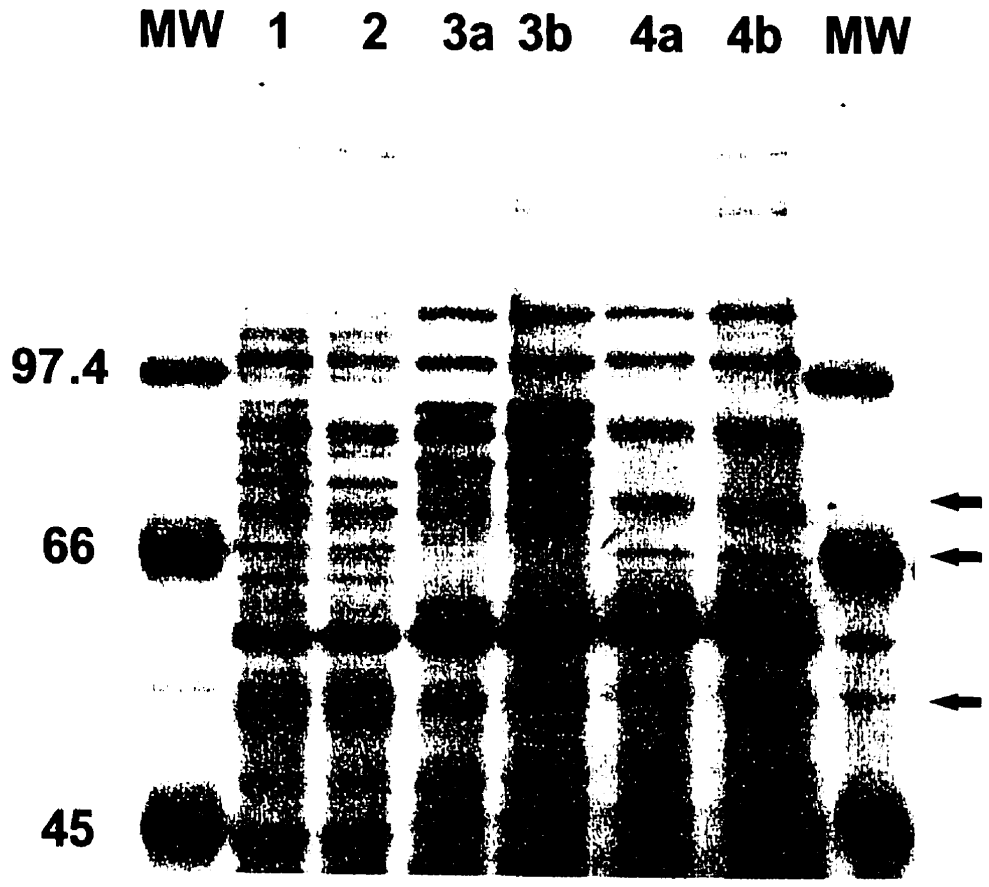

MW
1
2
3a
3b
4a
4b
MW
97.4
66
45

Figure A.2

Enrichment of vra-a.

Numbers to the left of the panels represent the apparent molecular masses of protein standards in kilodaltons. Lanes: MW, Bio-Rad protein standards; 1, BP338; 2, BP338 modulated; 3, BP347; 4, protein electroeluted from SDS-PAGE of BP347 OMVs.

A. Coomassie stained 16% SDS-PAGE.

B. Immunoblot probed with anti-vra-a antibody (14EG12E3).

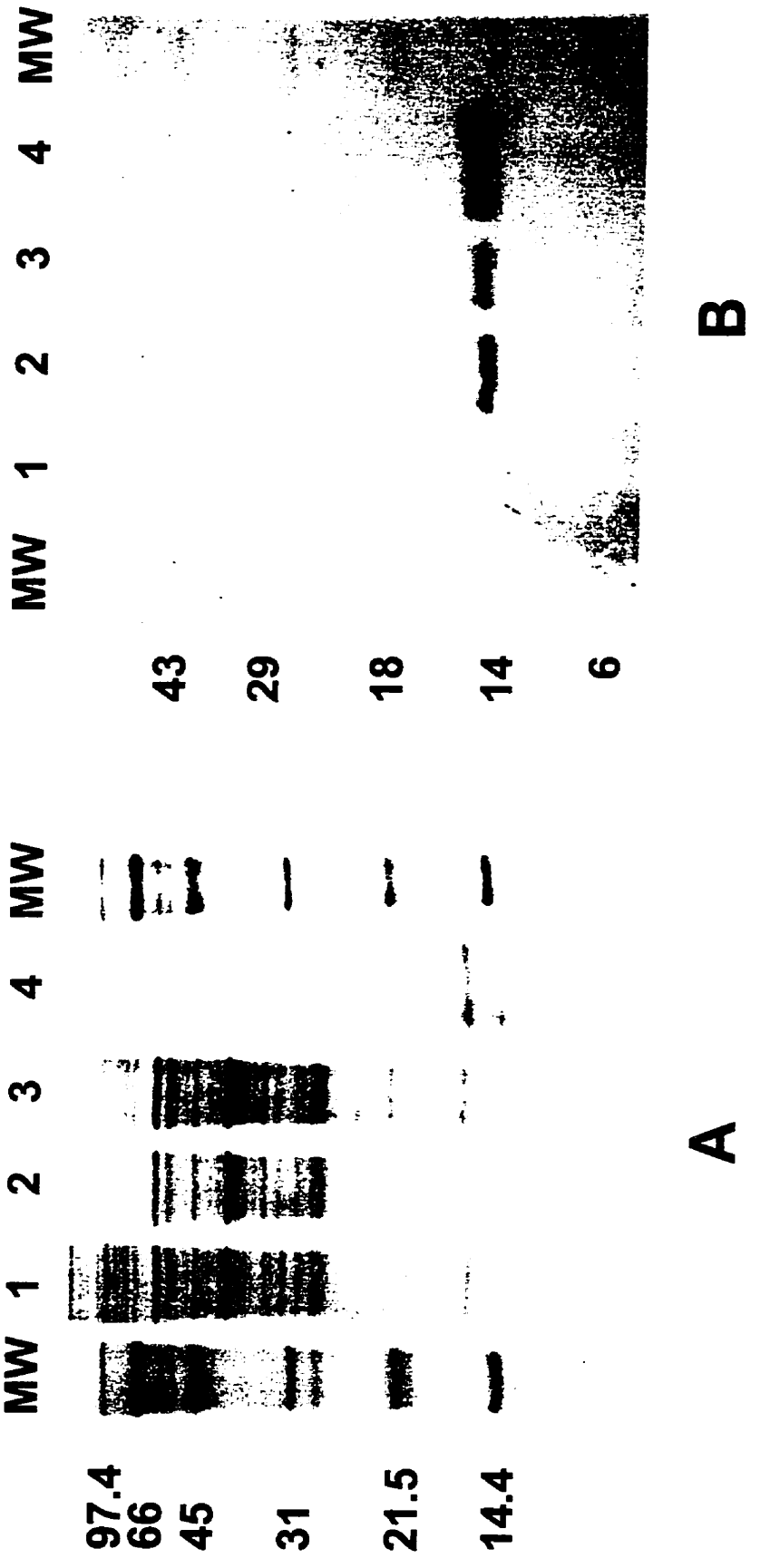

MW
1
2
3
4
MW
43
29
18
14
6
B
MW
1
2
3
4
MW
97.4
66
45
31
21.5
14.4
A

Figure A.3

Western immunoblot analysis of vra expression in two clinical strains.

Numbers to the left of the panels represent the apparent molecular masses of protein standards in kilodaltons. Lanes: MW, Gibco-BRL pre-stained low molecular weight protein standards; 1, UAH14797; 2, UAH14797 modulated (m); 3, UAH9775; 4, UAH9775m.

A. Immunoblot probed with anti-vra-a antibody (13A8B culture supernatant).

B. Immunoblot probed with anti-vra-b antibody (7H1A1 culture supernatant).

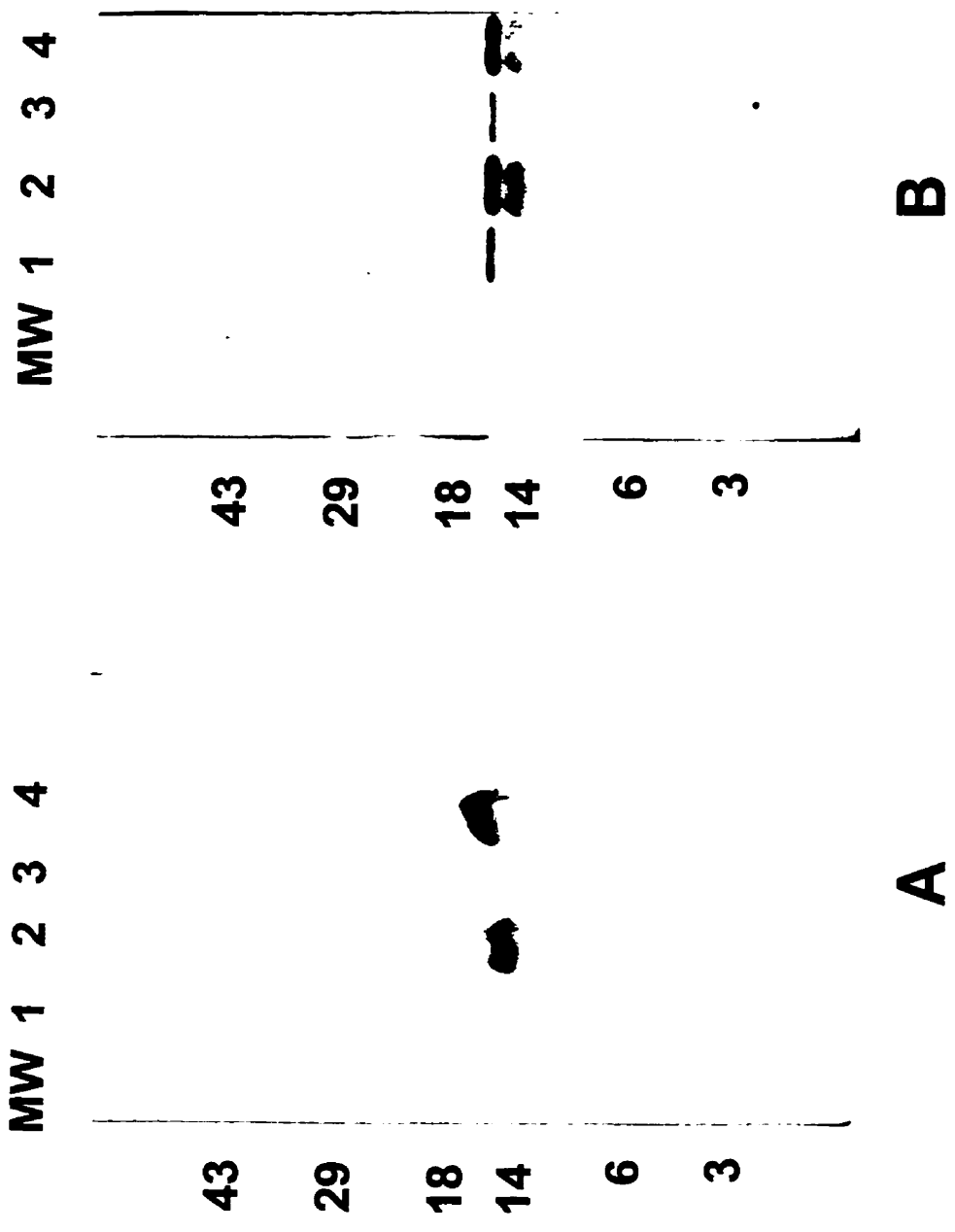

MW 1 2 3 4
43 29 18 14 6 3
B
MW 1 2 3 4
43 29 18 14 6 3
A

Figure A.4

Analysis of vra expression in 18323 *vrg*::Tn*phoA* mutants.

Numbers to the left of the panels represent the apparent molecular masses of protein standards in kilodaltons. Lanes: MW, Gibco-BRL pre-stained low molecular weight protein standards; 1, 18323; 2, 18323 modulated (m); 3, SK6; 4, SK6m; 5, SK18; 6, SK18m; 7, SK24; 8, SK24m: 9, SK53; 10, SK53m; 11, SK73; 12, SK73m.

A. Immunoblot probed with anti-vra-a antibody (13A8B culture supernatant).

B. Immunoblot probed with anti-vra-b antibody (7H1A1 culture supernatant).

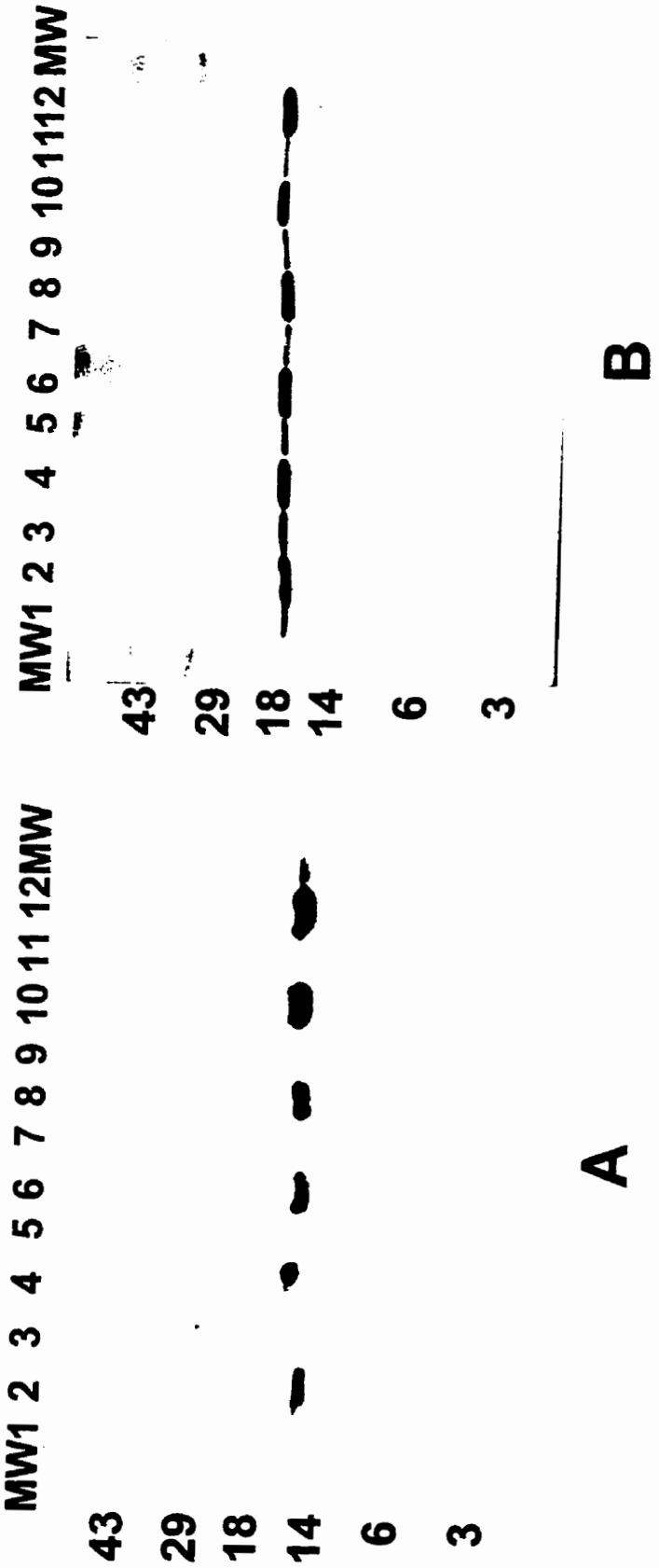

MW1 2 3 4 5 6 7 8 9 10 11 12MW
43
29
18
14
6
3
A
MW1 2 3 4 5 6 7 8 9 10 11 12 MW
43
29
18
14
6
3
B

Figure A.5

Proteinase-K digestion of vras.

Numbers to the left of the panels represent the apparent molecular masses of protein standards in kilodaltons. Lanes: MW, Gibco-BRL pre-stained low molecular weight protein standards; 1, BP338; 2, BP338 modulated (m); 3, BP338 proteinase-K digested; 4, BP338m proteinase-K digested.

A. Coomassie stained 16% SDS-PAGE.

B. Immunoblot probed with anti-vra-a antibody (13A8B culture supernatant).

C. Immunoblot probed with anti-vra-b antibody (7H1A1 culture supernatant).

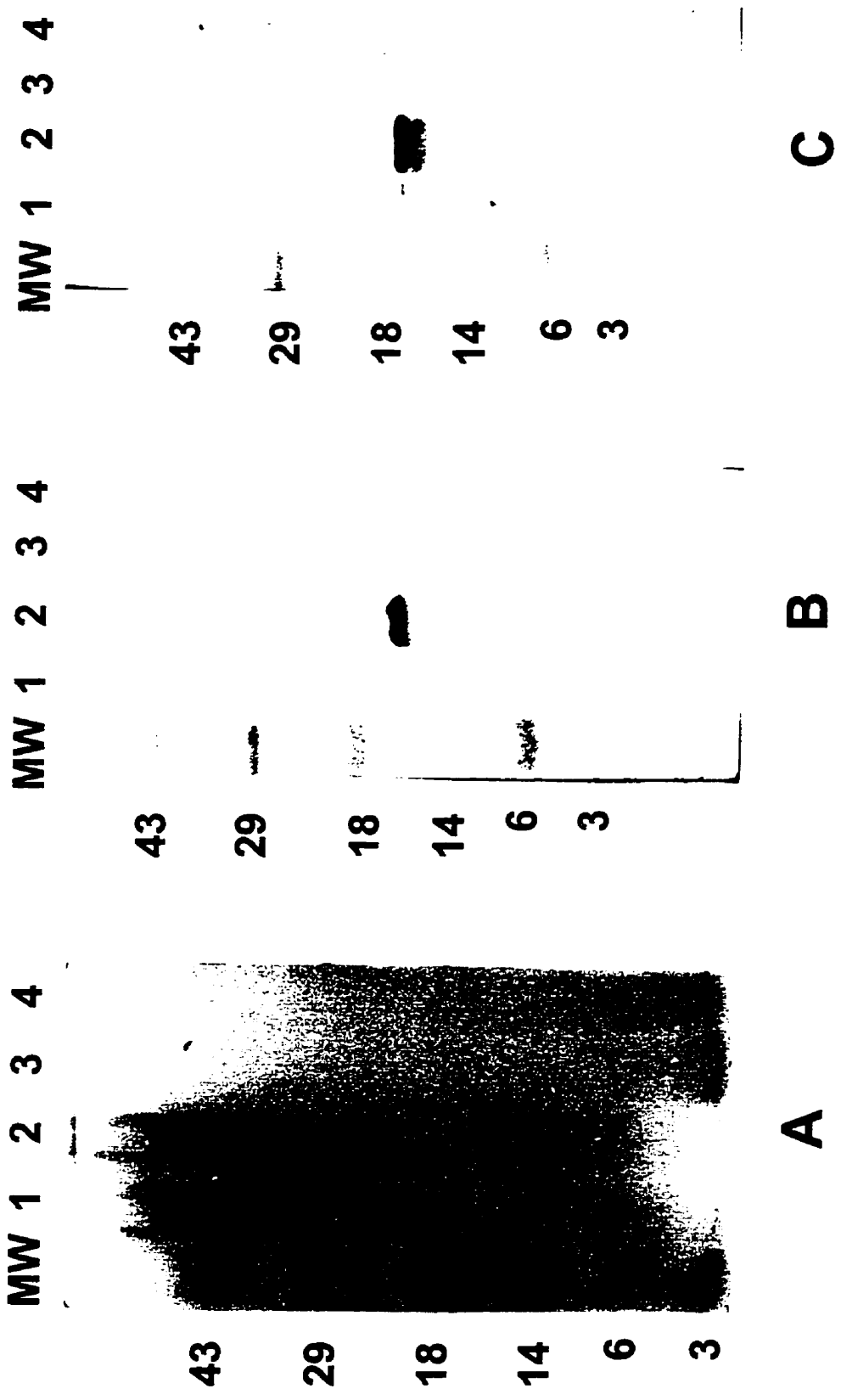
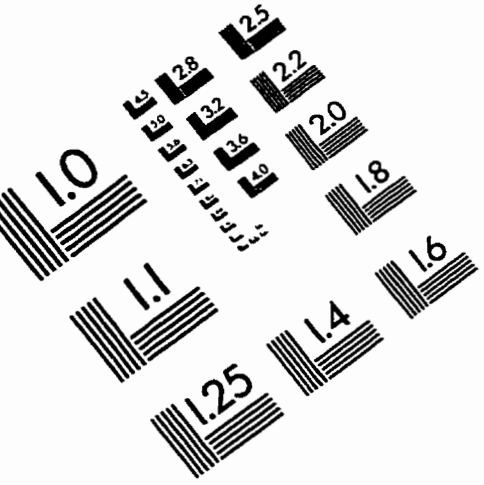
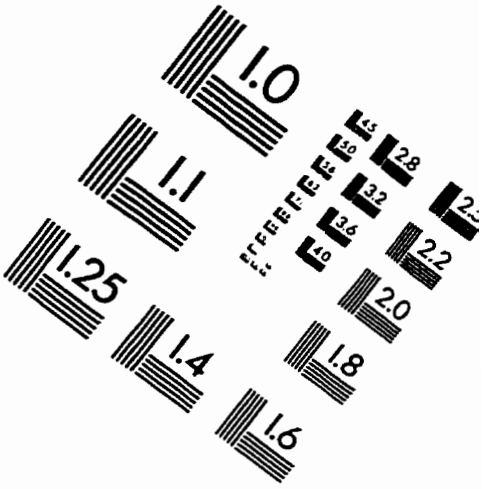
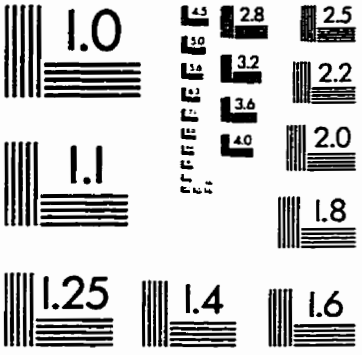
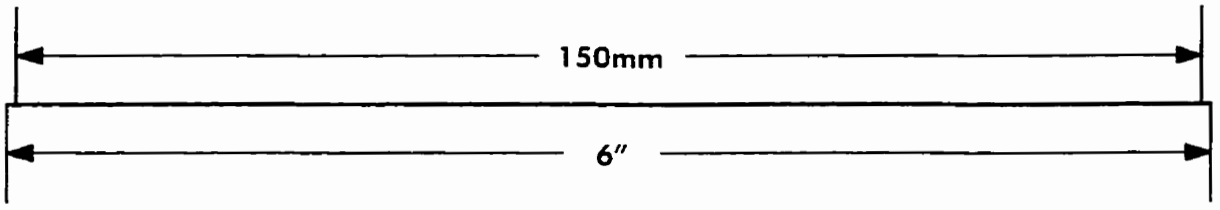

MW 1 2 3 4
43 29 18 14 6 3
A
MW 1 2 3 4
43 29 18 14 6 3
B
MW 1 2 3 4
43 29 18 14 6 3
C

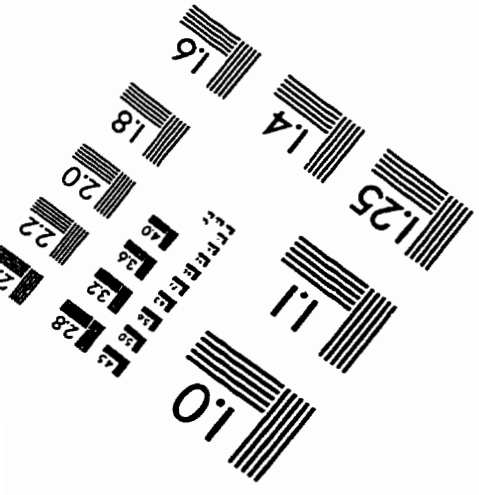
IMAGE EVALUATION
TEST TARGET (QA-3)

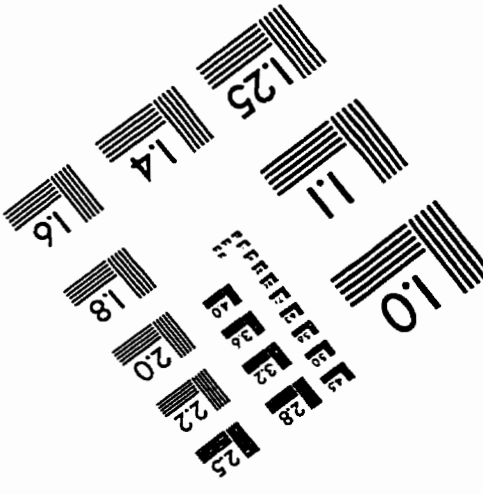












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