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University of Alberta

Identification of Modulation-Specific Genes in *Bordetella pertussis* using a GFP-Based screen

By

Regina Broitman-Maduro 

A Thesis

Submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of Master of Science

Department of Medical Microbiology and Immunology

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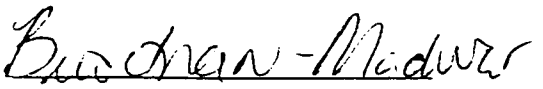

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Abstract

A Green Fluorescent Protein (GFP)-based promoter-trapping screen was developed to identify novel *bvg*-repressed genes in the human-only pathogen, *Bordetella pertussis.* A promoter library was created in the GFP expression vector pGB5 and screened for modulation-specific transgene expression. Five clones were identified by flow cytometry that expressed GFP under modulating conditions (pGB5-P2, pGB5-P5, pGB5-P7, pGB5-P9 and pGB5-P10). Of those, one clone, pGB5-P10, was the previously identified *vrg*6. Two novel clones, pGB5-P2 and pGB5-P9 were shown, through BvgA mutation, to have their expression controlled by the BvgAS regulon, but the remaining two were modulation-specific by another unknown mechanism. Clones pGB5-P2, pGB5-P9 and pGB5-P10 had diminished GFP expression when grown in high osmolarity broth and on Bordet-Gengou agar. This may implicate that other regulators, like OmpR may be involved in the modulatory cascade. Sequence analysis revealed homologues in other bacterial species to metabolic, stress-responsive genes and response-regulator genes. This may provide important clues to the *in vivo* modulatory niche for *B. pertussis.*

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Identification of Modulation-Specific Genes in *Bordetella pertussis* using a GFP-Based Screen submitted by Regina Broitman-Maduro in partial fulfillment of the requirements for the degree of Master of Science

Dr. M.S. Peppler

Dr. R.T. Irvin

Dr. L. Frost

31 August 2000

Dedication

For my parents, my sister and my husband who all helped and hindered this work in many special ways.

Acknowledgments

I wish to sincerely thank my supervisor, Dr. Mark Peppler, for showing me how exciting science could be and for encouraging me to "try anything once". I owe a debt of gratitude to my husband, Morris F. Maduro for helping me grasp the "bigger picture" and for helping me to avert experimental disasters (on several occasions). I wish to thank my committee, Dr. Laura Frost for being a tremendous mentor throughout my Undergraduate and Graduate training and Dr. Randy Irvin for teaching me humility. Thanks to everyone who encouraged me, my lab colleagues (especially Trevor Stenson and Christina Rogers), my family and friends- I may not have finished without your support.

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List of Abbreviations

2D-SDS-PAGE	two-dimensional sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis
AC	adenylate cyclase toxin
AMP	adenosine-mono-phosphate
ATP	adenosine-tri-phosphate
BGA	Bordet Gengou agar
Blast	basic local alignment search tool
BrkA	**B**ordetella **r**esistance to **k**illing- protein
bvg	***B**ordetella* **v**irulence **g**ene locus
BvgA	*bvg*-activator
BvgR	*bvg*-repressor
BvgS	*bvg*-sensor
CFU	colony forming unit
C-mode	cyanic mode
DFI	differential fluorescence induction
dNTPs	nucleotide tri-phosphates
DT	dermonecrotic toxin, heat labile toxin
FACS	fluorescence automated cell sorter
FHA	filamentous hemagglutinin
GFP	Green Fluorescent Protein
IL-1	interleukin-1

I-mode	intermediate mode
Kb	kilobase pair
LPS	lipopolysaccharide
mod	modulators (e.g. nicotinic acid and sulfate)
mRNA	messenger RNA
ORF	open reading frame
PCR	polymerase chain reaction
PT	pertussis toxin
REDOX	reduction-oxidation
RGD	arginine-glycine-gluatamic acid protein motif
rpm	revolutions per minute
SAP	Shrimp alkaline phosphatase
SSB	Stainer-Scholte broth
TCT	tracheal cytotoxin
T-TBS	Tris-buffered saline with tween-20
vir	Bordetella virulence locus, now called *bvg*
Vra	*vir*-repressed antigen
vrg	*vir*-repressed gene
X-mode	xanthic mode

1. Introduction

1.1 The Genus Bordetella

The Bordetellae are a group of obligate parasites that infect the respiratory tract of warm-blooded animals and can cause disease in both healthy and compromised hosts. There are currently six species described with different host tropism (Table 1) (Parton, 1999). *B. pertussis*, the etiological agent of whooping cough, is the only obligate human pathogen in the genus. As such, it has no known environmental reservoir (Gordon and Hood, 1951). *B. parapertussis* causes a mild form of whooping cough in humans, but is no longer considered to be a human-only pathogen as it has recently been cultured from healthy and pneumonic sheep (Porter *et al.*, 1994; van der Zee *et al.*, 1996). *B. bronchiseptica* is an important broad host-range veterinary pathogen that infects four legged mammals and birds with both acute and chronic illness (Gordon and Hood, 1951l). Two examples of acute illness caused by the organism are kennel cough in dogs and atrophic rhinitis in piglets (Goodnow, 1980). A long-term carrier state has been described for infected animals. For many, including the rabbits used for the *B. bronchiseptica* infection model, colonization with the organism does not always lead to clinical infection and depends on the health of the host (Cotter and Miller, 1994; Yoda *et al.*, 1982). Although seldom associated with human disease, *B. bronchiseptica* has been known to cause septicemia in compromised patients and is an opportunistic

Table 1. Bordetellae host specificities

Species	Host
B. pertussis	man
B. parapertussis	man, sheep
B. bronchiseptica	4-legged mammals, birds, man (rare)
B. avium	birds
B. hinzii	Birds, man (rare)
B. holmesii	man

pathogen (Libanore 1995) (Woolfrey and Moody, 1991). This is in contrast to B. *pertussis* and *B. parapertussis* for which disease is correlated with colonization of the organism in the respiratory tract (Linneman and Perry, 1977; Weiss and Hewlett, 1986). *B. avium* and *B. hinzii* are both avian pathogens that cause respiratory disease in turkey poults, chickens and other birds. Human isolates of *B. hinzii* have recently been identified and thus it has a broader host range than previously thought. *B. holmesii* identifies a group that has been cultured from human blood and appears to be a human-only pathogen. Both *B.hinzii* and *B. holmesii* were cultured from compromised patients and are considered opportunistic pathogens (Kersters *et al.*, 1984; Vandamme and Hommez, 1995).

Most Bordetellae have strict host and tissue tropisms. This can be correlated to the degree of fastidiousness among members of the group. Among the least fastidious are *B. bronchiseptica* and *B. avium,* which can survive and grow, for a limited number of replications, in phosphate-buffered saline. These organisms have been found in lake water and are able to grow in a wider temperature range than *B. pertussis* (10C-37C) (Porter, 1991). This may suggest an environmental reservoir for these organisms. *B. pertussis* and *B. parapertussis*, however, cannot grow in these conditions and have no known environmental reservoir. In contrast to *B. avium* and *B. bronchiseptica* they are the most fastidious members of the group and appear highly adapted to propagate and spread from human to human.

1.2 Clinical Disease

Bordetella pertussis is the small coccobacillus that causes the severe childhood respiratory affliction termed whooping cough. This gram-negative microbe infects upwards of 50 million people worldwide and is responsible for 0.5 million fatalities annually, most notably in third-world countries and among non-immunized children (Hewlett, 1990). The disease, named for the distinctive cough that is indicative of acute respiratory distress, manifests itself in three classical stages (Connor, 1981). The initial (catarrhal) phase marked by non-specific cold-like symptoms including a dry, non-productive cough occurs between 5-7 days after exposure to the organism through tiny air-borne aerosolized droplets or possibly direct contact with an infected individual (Weiss and Hewlett, 1986). During this phase it is possible to isolate the organism from nasopharyngeal cultures at a rate of 90%, as the bacteria are rapidly colonizing the respiratory tract (Weiss and Hewlett, 1986).

Following the initial stage of infection the progression to the paroxysmal phase is characterized by productive coughing spasms, which are the hallmark of the disease ("whoop") and are often accompanied by other adverse symptoms including hypoglycemia, weight loss, leukocytosis and rarely, neurological sequelae (Pittman, 1979). This stage occurs as the bacteria gain a stronghold in the lung alveoli and destroy the ciliated epithelia through adherence and directed intoxication (Tuomanen and Hendley, 1983). The pathophysiological manifestations of disease are thought to result from toxin dissemination (Pittman,

1979); Pittman 1984). This stage can last for several weeks before the patient enters the convalescent phase where the bacteria are cleared and no longer obtainable by nasopharangeal culture (Weiss and Hewlett, 1986). At this time the bacterium cannot be detected through standard nasopharyngeal culture methods, as they have been demonstrated in a murine pertussis model to be inside alveolar macrophages during the complaisant stage of disease (Cheers, 1969; Cheers *et al.*, 1971; Cheers and Gray, 1969; Gray and Cheers, 1969). This phenomenon has also been demonstrated in cultured alveolar macrophages *in vitro* (Saukkonen *et al.*, 1991). Although it is well established that intracellular bacteria constitute as much as half of the total number of bacteria early in the course of infection, there appears to be no pulmonary pathology associated with invasion in a rabbit infection model. Instead, extracellular *B. pertussis* that is bound to ciliated cells and macrophages contributes to pulmonary pathology. This may be consistent with a quiescent intracellular state that is compatible with survival inside human cells, although in a murine model intracellular bacteria that survive to the complaisant stage of disease are soon cleared (Saukkonen *et al.*, 1991).

1.3 Virulence Determinants

Bordetella pertussis is an obligate human-only pathogen with no known environmental reservoir (Gordon and Hood, 1951). As such, it is likely to be adapted for life within the human host. The manifestation of clinical symptoms during the disease progression is thought to be a direct result of a

developmentally regulated series of host-pathogen interactions (Table 2). Initially, it is thought that bacteria are delivered to the respiratory epithelia by aerosol droplets and must attach to ciliated cells in order to avoid clearance and establish infection. The intimate contact between bacteria and host cells is imperative not only at the start of infection, but also throughout the course of the disease. Adherence is required for colonization (initially) and persistence (Opremcak and Rheins, 1983).

B. pertussis has evolved numerous adhesins that are postulated to be involved in these critical step, including several afimbrial adhesins: filamentous hemagglutinin (FHA), pertactin, pertussis toxin (PT), **B**ordetella **r**esistence to **k**illing protein (BrkA) and four fimbrial genes (Brennan *et al.*, 1988; Fernandez and Weiss, 1994; Gross and Rappuoli, 1988; Sandros and Tuomanen, 1993; Stibitz and Miller, 1994; Willems *et al.*, 1990). Early *in vitro* tissue-culture studies suggested that FHA and PT were critical for attachment to human (ciliated) respiratory cells and mutants lacking either molecule were unable to attach (Tuomanen *et al.*, 1985). Subsequent animal studies confirmed that FHA mutants were impeded in the ability to persist in both the trachea and nasopharynx of mice (Kimura, 1990; Mooi, 1990). Indeed, a recent study examining all the major adhesins of *B. pertussis* has reported that FHA is the major factor in colonization of the entire respiratory tract (van den Berg, 1999). Biochemically, it was demonstrated that host-pathogen attachment was mediated through a carbohydrate molecule on the eukaryotic cell surface (lactosylceramide) and a carbohydrate recognition domain on the bacterial surface. The binding could be

Table 2. Role of Bordetellae Virulence determinants

Virulence Factor	Role in Pathogenesis
Tracheal cytotoxin	Ciliastasis, evasion of host defense
Fimbriae	Attachment, invasion
Filamentous haemagglutinin	Attachment, invasion
Adenylate cyclase	Evasion of host defenses, local damage
Pertactin	Attachment, invasion
BrkA	Attachment, invasion, serum resistance
Tracheal colonization factor	Attachment, invasion
Pertussis toxin	Attachment, invasion, interference with immune cells, systemic disease

impeded with a glycogonjugate containing galactose-N-acetylglucosamine (Tuomanen *et al.*, 1985; Tuomanen and Hendley, 1983; Tuomanen *et al.*, 1983). More recent data have suggested that FHA, BrkA and pertactin contain an arginine-glycine-glutamic acid (RGD) tripeptide sequence, the characteristic eukaryotic recognition motif for binding to host cell surface integrins (Finlay and Falkow, 1997; Relman *et al.*, 1990). Integrins are receptor molecules involved in cell adhesion. Specifically, they are expressed by various activated immune cell populations, including macrophages, and allow binding to vascular endothelial cells. This RGD sequence mediates FHA binding to the leukocyte integrin CR3, which mediates bacterial uptake into macrophages without triggering an oxidative burst (Relman *et al.*, 1990). Additionally, pertussis toxin (S2 subunit) and FHA share homology to alternate binding molecules, most notably human selectins, which like integrins, are cell adhesion molecules which mediate binding to both macrophages and ciliated cells (Sandros and Tuomanen, 1993). Thus *B. pertussis* is capable of binding to ciliated cells through a carbohydrate recognition domain and to immune cells, such as macrophages, via the RGD triplet moiety. Attachment to immune cells may be critical for toxin delivery and perhaps invasion.

Fimbrial genes, of which there are several in *B. pertussis*, are known to be important for mediating attachment to host cells in many bacterial species including *Escherichia*, *Haemophilus, Yersinia, Pseudomonas, Neisseria* and others (Krabbe *et al.*, 2000). It has been demonstrated in a murine pertussis model that fimbrial mutants are less well able to colonize the tracheas of mice,

however, colonization of the nasopharynx and lungs is not affected (Mooi 1992; Willems 1993). In addition, immunization with fimbriae can elicit protection in an animal model, suggesting that they are expressed during infection (Ashworth *et al.*, 1986). A recent study has reported that fimbriae are critical during the later colonization of laryngeal mucosa and as such are thought to be involved in persistence rather than initial colonization (van den Berg 1999).

The abundance of adherence molecules in this respiratory pathogen exemplifies the complexity and importance of bacterial adherence for the initial (catarrhal) and later (paroxysmal) stages of microbial pathogenesis. Although the numerous adhesins and complex regulation of these factors may seem to be redundant and unnecessary, they guarantee that the bacterium will adhere firmly, when needed, to mucosal surfaces or other target cells and are likely expressed at different times during infection for different outcomes.

As the bacteria adhere and multiply in the respiratory tract, both local damage to ciliated cells and systemic intoxication ensues (Muse *et al.*, 1979; Weiss and Hewlett, 1986). This is a result of the interplay between the host's immune response and several specific toxins produced by *B. pertussis* including: tracheal cytotoxin (TCT), pertussis toxin (PT), adenylate cyclase toxin (AC), and dermonecrotic toxin (DT). The toxins have a dual complementary purpose in that they cause damage to the host while simultaneously inhibiting the immune responses to damaged epithelium, thus allowing the bacterium to evade host defenses (Weiss *et al.*, 1984; Wolff *et al.*, 1984). These toxins act both early and

late during the paroxysmal stage of infection and are implicated in the pathology of whooping cough.

Ciliastasis (paralysis of beating cilia) and tissue necrosis are primarily caused by tracheal cytotoxin, which is delivered to ciliated cells by adherent bacteria. Interestingly, TCT is a non-protein muramyl peptide derived from peptidoglycan fragments that are constantly being shed. These fragments elicit a lethal up-regulation of the cytokine IL-1 that is ultimately responsible for a localized inflammatory response through the accumulation of acute phase proteins secreted by the host cells. This is not unlike the host's response to bacterial endotoxin (LPS) (Goldman *et al.*, 1982). The damage caused indirectly by TCT could help the bacteria to become established and grow in the respiratory tract. It has been hypothesized that during this early stage of infection, the bacteria avoid immune detection by impairing the killing ability of local immune-effector cells with adenylate cyclase toxin (Boschwitz, 1997; Gueirard, 1998). This enzymatic-toxin is able to enter phagocytic cells, convert intracellular ATP to cyclic AMP in the presence of the eukaryotic molecule calmodulin, and thereby disrupt normal cell functions. It has been demonstrated in tissue culture that once activated, AC will disrupt many immune cell processes including chemotaxis, phagocytosis, microbial killing and induce apoptosis (Collier *et al.*, 1977; Hewlett *et al.*, 1985; Slungaard *et al.*, 1983).

It is generally thought that systemic intoxication of patients with active whooping cough primarily results from pertussis toxin dissemination. Indeed, although clinical characteristics between *B. pertussis* and *B. parapertussis*, which

lacks PT, can be very similar, the severity of disease is greater when PT is present (Wirsing von Konig and Finger, 1994). In a coughing rat model, only *B. pertussis* strains with an intact PT could cause coughing paraoxysms, suggesting a role in cough production (Parton *et al.*, 1994). Unlike AC, which intoxicates surrounding cells, PT is disseminated and acts later during infection. PT, an ADP-ribosylating toxin, acts to uncouple numerous eukaryotic signaling pathways from their membrane receptors, thereby disrupting immune-cell migration and homing (Tamura *et al.*, 1982; Ui, 1988). Thus, the bacterium keeps certain immune cells away from the respiratory mucosa, where they are needed for microbial killing. This potent toxin is responsible for many of the symptoms associated with whooping cough (leukocytosis, weight loss, hyperinsulemia) and these manifestations of disease can be duplicated in a murine pertussis model with the perenteral administration of purified pertussis toxin (Pittman, 1980). Although critical for disease progression, PT cannot alone account for whooping cough symptoms as *B. parapertussis,* can cause similar disease without PT expression (Wirsing von Konig and Finger, 1994). Such observations are difficult to reconcile, however, it is likely that there exists co-operativety and redundancy amongst the toxins produced and they may contribute synergistically to the severity of disease.

DT (or Heat-Labile Toxin) causes dermonecrosis and has numerous local inflammatory effects. It is known to induce actin reorganization and multinucleation in eukaryotic cells (Pullinger *et al.*, 1996). DT is lethal when injected subcutaneously in mice, however, it is not required to establish infection

in the murine model and, as such, its role in pathogenesis remains unclear (Weiss and Goodwin, 1989). This may be due to the limitations of this animal model to illustrate the roles of certain virulence determinants outside the human host.

As the disease progresses and the patient enters the convalescent stage of infection, the bacteria can no longer be detected by culture in human lung alveoli. It is unknown whether they are still present in the human host at this time. This has led some to hypothesize that at this time the bacteria are cleared and the remaining malaise is due to toxin dissemination. In this view whooping cough is a toxin-mediated disease (Pittman, 1979; Pittman, 1984). It is well documented, however, that *B. pertussis* have the ability to invade various human tissue culture cell lines, including epithelial cells and macrophages (Everest *et al.*, 1996; Ewanowich *et al.*, 1989; Leininger *et al.*, 1992). It is possible that the bacteria avoid detection by invading immune cells. There has been much speculation as to whether the bacteria have an *in vivo* intracellular niche. Although this may explain how a human-only pathogen can insure long-term survival; the hypothesis remains unproven.

The majority of virulence determinants are shared amongst the members of the Genus Bordetella. *B. pertussis, B. parapertussis and B. bronchiseptica* all have a battery of functional toxins and adhesins to insure infection of their respective hosts (TABLE 3). The notable exceptions are pertussis toxin and tracheal colonization factor, which are present in *B. parapertussis* and *B. bronchiseptica* but are silent due to promoter mutations (Arico and Rappuoli,

Table 3. Bordetellae Virulence Factors

Virulence Factor	*B. pertussis*	*B. parapertussis*	*B. bronchiseptica*	*B. avium*
Tracheal cytotoxin	+	+	+	+
Fimbriae	+	+	+	+
Filamentous haemagglutinin	+	+	+	-
Adenylate cyclase	+	+	+	-
Pertactin	+	+	+	?
BrkA	+	?	?	-
Tracheal colonization factor	+	-	-	?
Pertussis toxin	+	-	-	-

1987). These factors are *B. pertussis*-specific and their activities have been attributed to the severity of disease caused by this organism.

1.4 Modulation-Specific Determinants

One intriguing aspect of *B. pertussis* biology is the ability of this organism to modulate virulence determinant expression in response to certain laboratory stimuli (Lacey 1960, Melton and Weiss 1993). When the organisms are grown at temperatures significantly below body temp (30^0C), or in the presence of chemicals like nicotinic acid, magnesium sulfate and B-vitamin derivatives, they shut off the expression of virulence determinants and turn on the expression genes that are collectively called *vrgs,* or modulation-specific determinants, (***vir***-**r**epressed **g**enes; the *vir* locus is now called *bvg,* and genes under the regulatory control of BvgAS are called *bvg*-repressed or *bvg*-activated genes. They will be referred to as such throughout this work) (Melton and Weiss 1989), (Lacey 1960) (Idigbe *et al.*, 1981; McPheat *et al.*, 1983). Although the major virulence determinants amongst the Bordetellae are shared and are similarly regulated, there exists great diversity in both the expression and control of *bvg*-repressed determinants (Akerley *et al.*, 1992; Beattie *et al.*, 1990; Giardina *et al.*, 1995; Knapp and Mekalanos, 1988; McMillan *et al.*, 1996)). At least 22 *bvg*-repressed protein bands are visible by two-dimensional sodium-dodecyl sulfate polyacrylamide gel electrophoresis (2D-SDS-PAGE) of modulated *B. pertussis,* although only a few *bvg*-repressed genes have been cloned and sequenced in

the literature (Beattie *et al.*, 1990; Beattie *et al.*, 1993; Stenson and Peppler, 1995; Weiss *et al.*, 1983; Weiss *et al.*, 1989). Among those are *vrg*6, *vrg*18, *vrg*24, *vrg*53, *vrg*73 which are Tn*phoA* mutants of genes whose products that have no homology to known proteins in other pathogenic bacteria (Beattie *et al.*, 1990; Beattie *et al.*, 1993). Others have been identified as surface-expressed proteins using modulation-specific antisera (Stenson and Peppler, 1995). In the literature there are reports of *bvg*-repressed gene induction between 2 and 10-fold under modulatory conditions, suggesting expression of these products in response to laboratory modulating stimuli (Beattie *et al.*, 1990; Weiss *et al.*, 1983). In cases where the *bvg*-repressed products have been identified, there have yet to be reports of sequence similarity to other known gene products in other organisms, which may give insight as to how and when they may be used in the life cycle of *B. pertussis*. Indeed, even between *B. pertussis and B. bronchiseptica* there exists great diversity in the *bvg*-repressed gene products despite the similarity of virulence determinant expression and regulation (Table 4). For example, only the expression of flagella, a siderophore and in some strains, ureases, have been described for the *B. bronchiseptica* modulated state. These proteins are absent in *B. pertussis,* although cryptic urease and flagellin genes are present in the organism (Akerley *et al.*, 1992; McMillan *et al.*, 1996; Moore *et al.*, 1995). In addition, *B. pertussis bvg*-repressed genes (*vrg6*, *vrg18*, *vrg24*, *vrg53*, *vrg73*) are not expressed in *B. bronchiseptica*; nor are the antigens Vra-A and Vra-B although at least one gene, *vrg6,* is cryptic in both *B. bronchiseptica* and *B. parapertussis* (Beattie *et al.*, 1990; Stenson and

Table 4. Bordetellae *Bvg*-repressed genes

Modulation-Specific determinant	*B. pertussis*	*B. bronchiseptica*
**vrg6, 18, 24, 53, 73*	+	-
***Vra-A, Vra B*	+	-
Urease	-	+
Flagella	-	+
Siderophore	-	+

*Bvg-repressed Tn-phoA mutants identified by Beattie et al, 1993

**Bvg-repressed surface antigens identified by cross-reactive monoclonal antibodies by Stenson and Peppler, 1995

Peppler, 1995). This group of proteins is a truly unique and elusive set of factors that are expressed under modulating laboratory conditions, but it remains unclear when, if ever, they are expressed *in vivo*.

1.5 Co-ordinate Regulation of *bvg*-activated and *bvg*-repressed genes

Most of the virulence determinants (with the exception of TCT) in *B. pertussis* are subject to co-ordinate and reciprocal regulation via the two-component regulatory system BvgAS (Arico *et al.*, 1989; Goldman *et al.*, 1982; Knapp and Mekalanos, 1988; Weiss *et al.*, 1983). This regulator is responsible for the co-ordinate expression of gene products that are required early or late during infection. Such control is important when trying to insure the timely expression and repression of critical proteins. Two regulatory states identified in early studies with the organism illustrate the nature of this co-ordinate response. The first, termed **phenotypic modulation,** is the freely reversible change that results in the down-regulation of virulence determinants and the de-repression of modulation-specific determinants in response to environmental conditions (Bordet and Sleeswyck, 1910; Lacey, 1960). It was noted in early work with the organism that the alteration of growth conditions by temperature and ionic media composition resulted in modulation of the organism into distinct "modes" (virulent to avirulent). These were based on antigenic characteristics and termed 1) X-mode (xanthic), 2) I- mode (intermediate) and 3) C-mode (cyanic)(Lacey, 1960).

The ability to modulate is the wild-type state for the organism, and insures that modulation specific gene products are never expressed during the virulent stage of infection. Modulation is common to members of the genus *Bordetella*.

The second, **phase variation**, is the result of a frameshift mutation by the insertion of a single base (G residue) in a string of 6 G residues in the coding sequence of *bvgS* (Arico *et al.*, 1991; Weiss and Falkow, 1984, Stibitz *et al.*, 1989). This results in the loss of virulence determinant expression and the gain of *bvg*-repressed gene expression, but is a genetically reversible change ($1X10^3$ - $1X10^6$ per colony forming unit)(Peppler, 1982). It should be noted that phase variants have been isolated from humans with increasing frequency during the later course of infection, although mutants of this sort are themselves unable to initiate infection in a murine model or to adhere to and invade cells in tissue culture (Kasuga, 1954; Martinez de Tejada *et al.*, 1998) (Ewanowich *et al.*, 1989; Friedman *et al.*, 1992; Lee *et al.*, 1990). In *B. bronchiseptica* phase variation is the result of a deletion in the coding sequence of *bvgS* and is thus nonreversible (Monack *et al.*, 1989).

As shown by transposon mutagenesis, regulatory control of virulence detrminants and modulation factors is dependent on a functional two-component regulator called BvgAS (Weiss *et al.*, 1983). The *bvg*AS loci amongst the *Bordetellae* are 96% homologous at the DNA level and respond to the same *in vitro* modulating signals, namely nictonic acid and sulfate anions, (Arico *et al.*, 1991). At the protein level, the few amino acid differences in BvgS result in a higher sensitivity to modulators for *B. bronchiseptica* relative to most *B. pertussis*

strains, as *B. bronchiseptica* modulates at significantly lower nicotinic acid and sulfate anion concentrations. The BvgA protein is identical amongst the species (Martinez de Tejada *et al.*, 1996). BvgAS mutants appear as phase variants and have lost the ability to modulate under laboratory conditions. As is typical for other two-component systems described, the regulon in *B. pertussis* consists of a sensor protein (BvgS) and a DNA binding response regulator (BvgA), which together sense environmental changes and orchestrate an appropriate response (Arico *et al.*, 1989; Stibitz *et al.*, 1989; Stibitz and Yang, 1991). (Figure 1.)

The sensor, BvgS, is a large, (1233 amino acid, 134 kDa,) cytoplasmic membrane protein with an environmental sensing N-terminal periplasmic domain (514aa) and four cytoplasmic domains which have effector function (Miller, 1992) The first cytoplasmic domain, called the **linker region,** is located adjacent to the second transmembrane domain and connects the periplasmic domain to the cytoplasmic effector domain. Next, the 232 aa **transmitter domain,** which has a conserved histidine residue and ATP binding site, is followed by two extra domains that are atypical of environmental sensors, but critical for BvgAS function, called the **receiver** and **C terminus**. BvgS is distinguished from other sensor proteins by these additional domains that are critical for functional sensing. Based on primary sequence homology with other sensor proteins it is predicted that the N-terminal periplasmic region senses specific environmental changes that result in a phospho-transfer cascade that begins at the transmitter domain and culminates in phosphorylation at the C terminus. Together these domains are responsible for enabling the autophosphorylation of the sensor

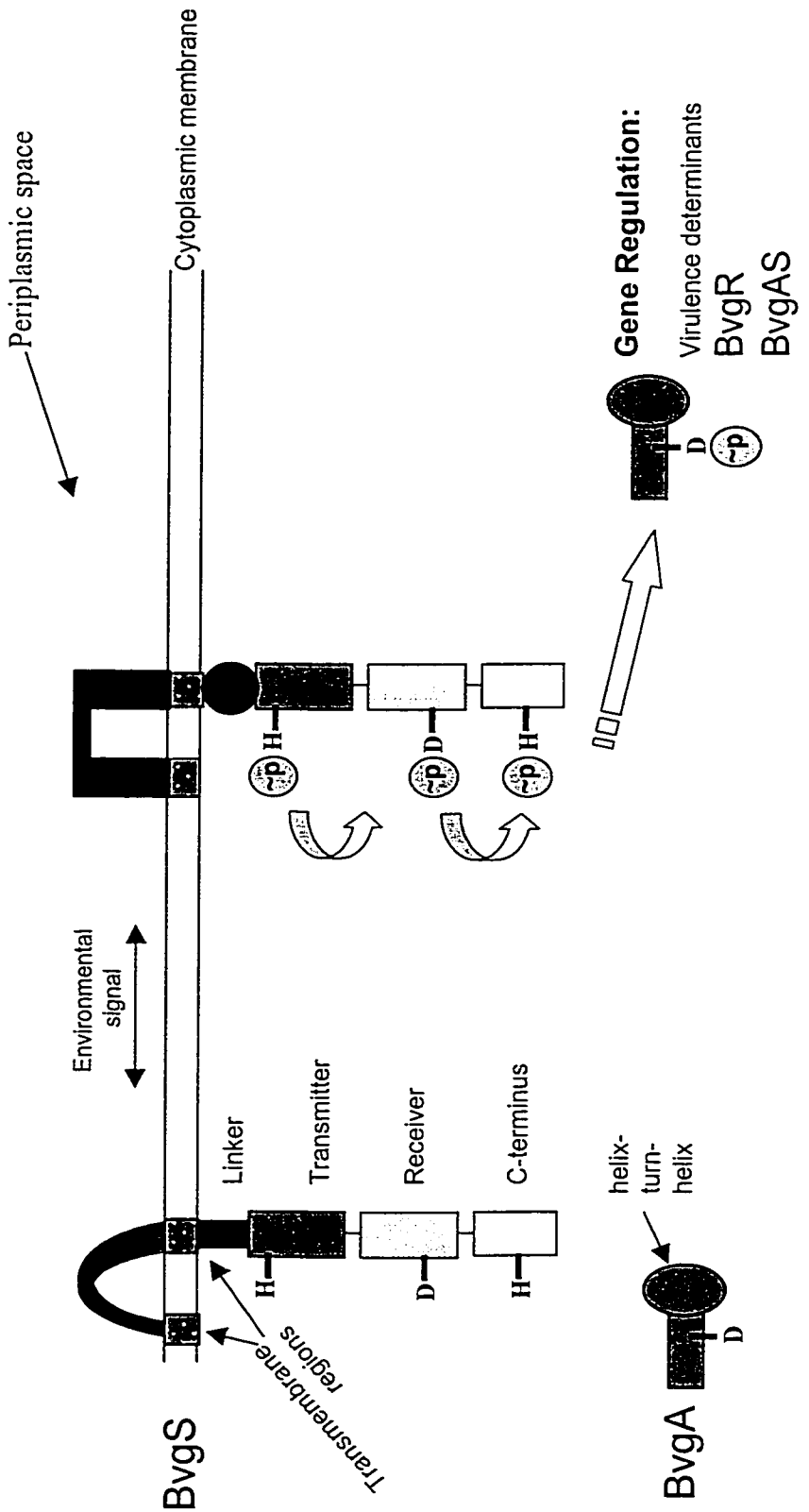


Figure 1. BvgAS signal transduction. Hypothetical steps in the BvgAS signal transduction phophorelay cascade. After an environmental signal is sensed, BvgS is in a conformationally active state and able to autophosphorylate at His-729 of the transmitter. An intermolcular transfer to Asp-1023 in the receiver culminates in transfer to the C terminus His-1172. BvgA is phosphorylated in a phosphotransfer reaction to Asp-54. In a phosphorylated state, BvgA is able to activate transcription of virulence genes through increased DNA binding affinity.

protein that begins at a histidine (H-729) in the transmitter domain which is transferred to an aspartate (D-1083) residue in the receiver and finally to another conserved histidine (H-1172) within the C terminus of the sensor molecule (Arico *et al.*, 1991; Boucher *et al.*, 1994; Manetti *et al.*, 1994; Uhl and Miller, 1994; Uhl and Miller, 1996a; Uhl and Miller, 1996b).

The response regulator, BvgA, is a 209 aa, 23kDa, protein that contains a receiver domain and a DNA binding domain of the helix-turn-helix motif (Arico *et al.*, 1989; Gross *et al.*, 1989). It is responsible for aiding transcriptional activation upon binding to specific DNA sequences, (the consensus being TTTCCTA), that are found in the regulatory regions of BvgAS responsive genes (Roy and Falkow, 1991). Binding of BvgA to these sites requires the phosphorylation of a conserved aspartate (D–54) residue in the receiver domain of BvgA, by BvgS, following the initial phopho-cascade. Phosphorylation of the aspartate residue in BvgA allows for a higher affinity interaction between the response regulator and target DNA (Boucher *et al.*, 1994).

It has been speculated that the temporal regulation of *bvg*-activated virulence determinants can be attributed almost entirely to the affinity of BvgAS-responsive promoters for phosphorylated BvgA, which is ultimately related to the amounts of the molecule in the cell (Prugnola *et al.*, 1995; Stibitz and Miller, 1994). Among the earliest virulence determinants to be transcribed are *fhaB*, *fim* and *bvg* itself. Transcription of these genes occurs minutes after a shift from modulating to non-modulating conditions (Scarlato *et al.*, 1991). This is not surprising given that the adhesins, filamentous haemagglutinin and fimbriae are

critical for the initial stages of infection and that continued BvgAS expression will insure that the bacteria can adequately control virulence gene expression. In contrast, activation of pertussis toxin (*ptx*) and adenylate cyclase toxin (*cya*) occur several hours after a shift to non-modulating media (Scarlato *et al.*, 1991; Scarlato and Rappuoli, 1991). In addition, these promoters appear to be more sensitive to the reciprocal conditions than *bvg* and *fha* and turn off sooner under modulating conditions. It has been demonstrated that *ptx* expression requires an excess of phosphorylated BvgA compared with *fhaB* and *bvg*. This suggests that the activation of certain promoters depends not only on the phosphorylation of BvgA but also on the amounts of phosphorylated response regulator in the cell (Uhl and Miller, 1995). Although it is likely that additional regulatory control mechanisms exist to allow the temporal expression of virulence determinants in Bordetellae, including accesory proteins that may alter superhelical density and promoter accessibility, it is clear that a major player is BvgAS(Dorman, 1991).

In contrast to the common mechanism of virulence determinant regulation by a typical two-component system, there exists an atypical repressor molecule in *B. pertussis* that is not found in other *Bordetellae*. The *bvg*-repressor (BvgR), is a 291 aa, 34kDa DNA binding protein responsible for insuring the reciprocal regulation of *bvg*-repressed genes and *bvg*-activated genes by repressing *bvg*-repressed gene expression during times when *bvg*-activated genes are expressed. BvgR is itself a *bvg*-regulated product and is transcribed when BvgAS is phosphorylated. It binds to *bvg*-repressed promoters at a common cis-acting regulatory site (the consensus being GT_ G_

TGCCGGC___G_CGCTGC___GC_CAT) and thereby impedes transcription(Merkel and Stibitz, 1995).

Although the two-component sensor/response-regulator proteins are fairly common amongst bacterial species, the additional response regulator, BvgR adds complexity to the BvgAS regulatory system in *B. pertussis* (Volz, 1995). In other *Bordetellae*, repression of *bvg*-repressed genes must occur by a different mechanism. For *B. bronchiseptica*, it has been shown that BvgA represses flagellar expression directly by binding to *frlAB* (Akerley and Miller, 1993; Akerley *et al.*, 1992). This controls the regulatory cascade normally initiated by *frlAB* expression and silences the operon. Thus in *B. bronchiseptica*, repression involves the modulation of the activator FrlAB and not the binding of a direct repressor like BvgR in *B. pertussis*. Presumably for other *Bordetellae* there exists similar or additional mechanisms for controlling *bvg*-repressed genes in the absence of signal. These mechanisms may include direct regulation of activators through BvgAS or may involve other regulators such as histone-like proteins, sigma factors, additional DNA binding proteins and even other response regulators. These aspects are in need of further study.

1.6 The Secret Life of *B. pertussis*

The presence and continued maintenance of the *bvg*-repressed genes in *B. pertussis* suggests that these products are utilized at some point during the developmentally regulated states of the organism. It is intriguing to speculate

when that may occur. It is known that this human-only pathogen requires virulence determinants during disease progression in order to infect a human host, which would preclude *bvg*-repressed gene expression, by the reciprocal regulation of these genes. This pathogen has never been associated with another niche where virulence determinants may be off. In addition, experiments done in a murine infection model have revealed that mutants locked in the modulated state were impaired in establishing infection (Akerley *et al.*, 1995; Martinez de Tejada *et al.*, 1998; Martinez de Tejada *et al.*, 1996). This has led some to the conclusion that *bvg*-repressed genes are never expressed *in vivo* and indeed they have been described as a "vestigial arm" of evolution that is no longer needed for *B. pertussis* (Martinez de Tejada *et al.*, 1996). Unfortunately, these conclusions are based on experiments done with strains of *B. bronchiseptica*, which is a markedly different organism. Martinez de Tejada *et al.* have speculated how *B. bronchiseptica* may require flagella for motility when outside a host in nutrient limited conditions and how flagella may be detrimental to a virulent organism that is expressing adhesins. For *B. pertussis*, however, such speculation is less straightforward. Experiments done with *B. pertussis* phase-locked mutants (modulated state) revealed they were impaired for infection. This is not surprising given that the organism needs to be virulent in order to establish infection. In addition, the inappropriate expression of *bvg*-repressed genes during infection does interfere with the bacterium's ability to cause disease in a murine model. This would indicate that the *bvg*-repressed genes are not necessary and even detrimental during the initial stages of

disease(Merkel *et al.*, 1998). However, assessing the role of modulation later in the course of disease is harder to test. This requires a wild-type bacterium that is able to respond to environmental stimulus and a relevant animal model.

Some have suggested that *bvg*-repressed proteins may aid in long term survival of the organism in an asymptomatic "carrier-state", thus insuring survival for the human-only pathogen. Although this is an intriguing hypothesis, a carrier state has never been found for *B. pertussis*. *B. bronchiseptica*, however, does have a well described carrier state and infected animals maintain the organism but show no signs of illness (potentially) for life (Yoda *et al.*, 1982). It is unknown whether this state involves *bvg*-repressed gene expression. The real trouble with proving an asymptomatic carrier state for adults is finding the organisms in the later stages of infection. It is known that as disease progresses it is increasingly difficult to culture the organism by nasopharyngeal swab, as organism are likely deep in the lung alveoli. To find a specimen may require broncholavage, or biopsy of the lungs, both invasive procedures in an otherwise healthy potential carrier. Although it may be possible to find volunteers for this procedure, a comprehensive study of this sort has not been undertaken. One aspect that seems to fit with a carrier state for *B. pertussis* is the observation that virulent cells are invasive in a tissue culture model. It is well documented that *B. pertussis* has evolved the ability to invade and survive in human epithelial cells and immune-effector cells, including macrophages *in vitro* at least for several hours (Ewanowich *et al.*, 1989; Friedman *et al.*, 1992). In addition, *in vivo* broncholavage samples from immuno-compromised children has revealed

intracellular organisms in macrophages (Bromberg *et al.*, 1991). Although the organism does not breach the primary site of infection and reach deeper tissue, it has been speculated that this invasive ability may allow for long-term survival within human cells. It seems consistent that modulation may occur inside host cells since the organism is necessarily virulent in order to attach and invade cells, but may need to turn off toxins and adhesins once inside so as not to kill the cell. Due to the reciprocal nature of *bvg*-activated and *bvg*-repressed gene regulation, this would necessarily mean that *bvg*-repressed gene expression would ensue once *bvg*-activated gene expression is down regulated. Interestingly, for *B. bronchiseptica* phase variation to the avirulent state has been shown to allow long term survival in professional phagocytes by giving the strains a survival advantage (Banemann and Gross, 1997). However, in marked contrast to *B. pertussis, B. bronchiseptica* does not require virulence genes for invasion in epithelial and dendritic cells (Guzman *et al.*, 1994; Schipper *et al.*, 1994). It should be noted that the non-requirement for virulence determinants makes it trivial to assess long-term survival for modulated *B. bronchiseptica.* The same experiment cannot be done for *B. pertussis*, as non-virulent bacteria are non-invasive. Caution should be exercised when assessing the role of *bvg*-repressed genes in other *Bordetellae* and extending the results to *B. pertussis*, as this human-only pathogen is different from the others in terms of virulence determinant expression, *bvg*-repressed gene expression, fastidiousness and pathogenesis. It is not comparable to simply mutate a modulation-specific determinant and assess the virulence of *B. pertussis* in a murine model without

some indication as to the function of that determinant in another organism with a more relevant animal model. The lack of an appropriate animal model makes it impossible to address many questions that have been explored for *B. bronchiseptica*, however, as more *bvg*-repressed products are cloned and identified, sequence homology to other known determinants of human respiratory pathogens may illustrate a possible role for these products in *B. pertussis*.

1.7 Purpose, Rationale and Goals of this Thesis

Our laboratory has been interested in understanding the *bvg*-repressed state of *B. pertussis* in the hopes of elucidating when modulation might be important for the organism. Prior to this work only a few *bvg*-repressed genes had been identified and cloned, but no significant homology to other factors was shown (Beattie *et al.*, 1993). All but one had a downstream *cis*-acting sequence that was required for proper regulation. This became a common element among this group of genes, that could be exploited in a screen to find more such determinants.

It became apparent that if a role for modulation was to be discovered, more modulation-specific gene products would be needed to give insight about regulation, expression and homology amongst other proteins of other pathogenic, invasive organisms. In order to help our understanding of modulation, a novel "promoter-trapping" screen to capture modulation-specific promoters was developed. This could potentially identify novel *cis*-acting elements that are

required for modulation-specific expression as well as revealing traditional *bvg*-regulated promoters. The hope was to identify additional gene products under modulatory control. Two novel applications of technology allowed such a screen to be developed. First, the utility of the fluorescence automated cell sorter (FACS) allowed for the rapid enumeration of thousands of bacteria per second based on size and fluorescence (Cormack *et al.*, 1996; Valdivia and Falkow, 1996). It was advantageous to develop a screen that utilized the flow cytometer, which could enumerate large sets of bacteria and generate statistically sound data that left little room for human error. Another advantage of using FACS was the single bacterium method of enumeration and analysis employed by the cytometer, which was beneficial for establishing expression trends for the culture population in terms of standard or skewed distributions. For example, the histogram generated by the flow cytometer enabled one to distinguish between a bacterial population where only a subset of clones are expressing high levels of GFP *vs.* an entire population expressing a normal distribution. This was not possible using standard colorimetric detection assay (β-galactosidase) which analyzes an entire culture at once. A recent study which compared relative promoter activity in *E. coli* using β-galactosidase detection and GFP fluorescence, concluded that the two were equally sensitive at detecting transcription from previously well-characterized promoters. However, it was also found that GFP based detection was better for agar based studies and had the added advantage that bacteria did not need to be killed during the assay (Lissemore *et al.*, 2000).

Second, the advent of a FACS-optimized Green Fluorescent protein (GFP) from *Aequorea victoria* allowed for high sensitivity in the flow cytometer without which the screen would have been less quantitative (Chalfie *et al.*, 1994; Cormack *et al.*, 1996; Inouye and Tsuiji, 1994; Prasher *et al.*, 1992). The use of GFP as a reporter was wide spread in eukaryotic biology, but its use as a reporter in prokaryotes was novel and preliminary (Valdivia and Falkow, 1996; Valdivia *et al.*, 1996). Luckily, this soluble, non-toxic protein proved to be easily expressed and properly folded in many bacteria (Chalfie *et al.* 1994, Valdivia *et al.* 1996). It should be noted that vast over-expression of many different proteins in prokaryotes results in protein sequestration to inclusion bodies and poor growth for the bacterium, however, the vast majority of studies report no problems with toxicity of GFP in numerous eukaryotic and prokaryotic systems (Justus and Thomas, 1999; Liu *et al.*, 1999; Margolin, 2000) (Weingart *et al.*, 1999). In addition, for the purpose of identifying novel *bvg*-repressed genes there was an established precedent of low expression from those genes already identified (Knapp and Mekalanos, 1988). This would make GFP inclusion body sequestration an unlikely outcome of promoter activity. Further, fluorescence of GFP has been demonstrated *in vitro,* indicating that chromophore formation is intrinsic to its polypeptide sequence and therefore requires no cellular machinery for folding and fluorescence. (Nishiuchi *et al.*, 1998; Reid and Flynn, 1997). Finally, *in vivo* fluorescence of GFP within the context of a heterologous polypeptide (i.e, a translational fusion, or GFP tag) has been demonstrated repeatedly in many systems, most relevantly in bacteria (Feilmeier *et al.*, 2000;

Margolin, 2000; Stretton *et al.*, 1998; Wehrl *et al.*, 2000). A recent collaboration between our laboratory and that of Dr. A. Weiss (University of Cincinnati, OH) has found that GFP is actually advantageous over external fluorescent antibody labels for phagocytosis studies, suggesting that expression of high levels of GFP is compatible with normal cell function (Weingart *et al.*, 1999). Therefore, if transgene *cis*-acting sequences are considered equal, and messages encoding GFP are translated at equal efficiencies, fluorescence as a function of GFP fusion protein accumulation should be proportional to promoter strength. In practice, GFP has been used successfully as both a transcriptional and translational reporter in nearly all systems amenable to study; bacteriophage (Rubinchik *et al.*, 2000), bacteria (Feilmeier *et al.*, 2000), yeast (Tatebe *et al.*, 2001), nematodes (Fukushige *et al.*, 1999), fruit flies (Lu *et al.*, 1999), sea urchins (Lu *et al.*, 1999), amphibians (Moritz *et al.*, 1999), and mammalian systems from mice to human cells (Perry *et al.*, 1999) (Dabiri *et al.*, 1997).

Before GFP reporters, it was possible, of course, to do similar screens for specifically induced promoters. Early work to identify *bvg*-activated genes and *bvg*-repressed genes in *B. pertussis* utilized transposon fusions to the coding region of β-galactosidase (*lacZ*) and alkaline phosphatase (*phoA*) as reporters for gene expression (Weiss *et al.* 1989, Beattie *et al.* 1993). Although both approaches were successful in generating a large bank of mutant bacteria, they were each cumbersome and limited in specific ways. The β-galactosidase fusions are still utilized today but require that bacteria be grown to a specific density (0.4) in liquid culture supplemented with 4% glucose, 1mM $MgSO_4$ and

vitamin B1, and treated extensively before colorimetric measurement with Z buffer (Miller 1972). Following color development the A_{600}, A_{420} and A_{550} are read to determine β-galactosidase activity through the following equation: 1000 X (A_{420} – 1.75 X A_{550})/ t X 0.1 X A_{600}, where t is the time of development in minutes. The assay cannot account for the proportion of bacteria that are expressing the enzyme, nor can the growth conditions be manipulated for effects on transgene expression. This assay can be error-prone and is particularly sensitive to reagent quality, as the color development relies on the buffer conditions and the substrate turnover (Maniatis, 1982).

The alkaline phosphatase assay is equally cumbersome, particularly since it requires that bacteria be grown in the presence of high followed by low phosphate concentrations to induce the enzyme, and then be replica plated onto nitrocellose prior to treatment with various reagents to generate color development (Beattie *et al* 1992). In contrast, GFP-expressing bacteria can be grown under various conditions and can be analyzed without adding any reagents or killing the organisms.

Together, the advent of a FACS-optimized GFP coupled to the power of the flow cytometer have aided us in the rapid identification of 4 novel modulation-specific genes, 2 of which do not appear to be under *bvg*AS control. Armed with new modulation-specific determinants, as more genomes are sequenced, it will be possible in future studies to address whether these genes are likely to be required during the later course of infection by comparing sequence homology to other known virulence determinants.

In this thesis I will describe:

I. The development of the "promoter trapping" screen to identify novel modulation-specific genes in *B. pertussis*

II. The GFP induction kinetics for five modulation-specific clones grown in agar, broth and high osmolarity media.

III. The identification and sequence analysis of five modulation specific determinants

IV. The evidence for alternate regulators involved in modulation specific induction.

V. The utility of modulation-specific GFP reporters for *in vivo* expression studies.

2. Materials & Methods

2.1 Bacterial strains, plasmids and media.

B. pertussis, *E. coli* and plasmid strains used in this study are presented in Table 5. All *B. pertussis* strains were routinely grown at 37°C with 98% humidity on Bordet Gengou agar (BGA; Difco Laboratories, Detroit, Mich.) containing 1% glycerol and 15% defibrinated sheep blood. Antigenic modulation was induced on BGA or in Stainer-Scholte broth (Stainer and Scholte, 1970) with 5mM nicotinic acid and 20mM $MgSO_4$ (BGA with modulators and SSB with modulators respectively) (Beattie *et al.*, 1990). For growth on agar, a quarter plate of a 3-day-old culture was used to streak three similarly sized areas on a fresh BGA plate. For growth in broth, a 2-3 day old broth-grown culture, shaken at 100 rpm, in a fluted flask was used to inoculate fresh Stainer-Scholte broth to an optical density of 0.05 at 540nm, in triplicate. All broth cultures were grown at 37°C in 6-well tissue culture trays in a maximum volume of 4 ml, with gyratory shaking at 100 rpm, except when used in osmolarity studies. The trays were routinely placed in sealed Tupperware containers lined with wet paper towels to maintain humidity. Cultures for osmolarity studies were grown statically in Stainer-Scholte broth with 10% sucrose as described above. Unless otherwise noted, antibiotics were used in

Table 5. Strains and plasmids used in this study

Species, Strains and Plasmids	Characteristics	Source or reference
***E.coli* K-12**		
DH5α	High-efficiency transformation	BRL
XL1-Blue	High-efficiency transformation	Stratagene
B. pertussis		
BP338	Wild-type, Naladixic acid resistant clinical isolate Tohama-I	Dr. A. Weiss (Weiss and Hewlett 1983)
BP337	BP338 bvgS1:Tn*5*	Dr. A. Weiss (Weiss and Hewlett 1983)
BP907	BP338, Str^r, ΔBvgA	Dr. S. Stibitz (Stibitz and Yang 1991)
BP536	BP338, Str^r,	Dr. S. Stibitz (Stibitz and Yang 1991)
Plasmids		
pBluescript KS^-	General cloning vector, Ap^r	Stratagene
pMM065	Derivative of pBluescript with *Bgl* II-*Eco* RI in polylinker	Dr. M. F. Maduro (Maduro 1998)
pGFP2-pKen	GFP high copy phagemid expression vector	Dr. B. Cormack (Cormack, Valdivia et al, 1996)
pMM065-GFP	GFP *Bam* HI-*Bgl* II fragment in pBluescript	This study
pGB3	*Bam* HI *vrg* 6 promoter fragment in pBluescript	This study
pGB4	GFP *Bam* HI-*Eco* RI fragment in pBluescript	This study
pBBR1MCS-2	Broad host range cloning vector, Kan^r	Dr. M.E Kovach (Kovach 1995)
pGB5	Derivative of pGB4 and pBBR1MCS-2	This study
pGB5-3	Non-functional *Bam* HI *vrg*6 fuison from pGB3 to pGB5	This study
pGB5-6	Colinear *Bam* HI *vrg*6 fusion in pGB5	This study

Table 5 continued. Strains and plasmids used in this study

Species, Strains and Plasmids	Characteristics	Source or reference
Plasmids		
pGB5-P1-P10	Random BP338 *Sau* 3AI fragments cloned into pGB5	This study
pGem T-easy	PCR T/A cloning vector	Promega
pGB7	pGB5-P2 cloned fragment in pGEM vector	This study
pGB9	pGB5-P7 cloned fragment in pGEM vector	This study
pGB17	pGB5-P10 cloned fragment in pGEM vector	This study
pGB19	pGB5-P5 cloned fragment in pGEM vector	This study
pGB20	pGB5-P9 cloned fragment in pGEM vector	This study

the following concentrations: kanamycin sulfate, 10 μg/ml; ampicillin, 50 μg/ml; naladixic acid, 50 μg/ml; streptomycin sulfate, 100 μg/ml (Fischer). *E. coli* strains were grown aerobically (with shaking at 180 rpm) in Luria Bertani (LB)(Miller, 1972) broth or agar, both incubated at 37°C

2.2 Recombinant DNA methods.

DNA manipulations were done according to standard protocols (Maniatis, 1982). Purification of plasmid DNA was achieved using Qiagen columns (Qiagen Inc, Mississauga ON, Can) or by standard alkaline lysis. Bands of interest were purified using Qiaex II beads (Qiagen) or by agarase digestion from 0.7% low-melt agarose. Ligations were allowed to proceed at 4°C for 24-48 hours. Plasmids were introduced into competent *E.coli* XL1B (Statagene) or DH5α (Bethesda Research Laboratories) by transformation, and into *B. pertussis* by electroporation (see section 2.5 below). Restriction endonucleases, T4 DNA ligase, tRNA and *Taq* polymerase for polymerase chain reaction (PCR) were purchased from either New England Biolabs or Bethesda Research Laboratories and used according to manufacturer's instructions. Shrimp alkaline phosphatase (SAP) was purchased from United States Biochemical (Arlington, II). A ThermoSequenase kit from Amersham was used for manual sequencing. Custom oligonucleotides were purchased from Bioserve Biotechnologies Ltd, (Laurel, MD).

2.3 Strain and plasmid construction

The general cloning strategy to construct the GFP expression vector is outlined in Figure 2. A 750-bp *Bam*HI-*Pst*I fragment from GFP2-pKEN (a gift from B. Cormack, Stanford University) encoding the green fluorescent protein (GFP) from *Aequorea victoria*) was cloned into the same sites of pDP#MM065 (M. Maduro) to create a convenient template for PCR, pMM065-GFP. GFP was amplified from pMM065-GFP using the primers MMA6: 5' CCC ATT ATC GAT AAG ATC TCC ACG GTG GCC 3' and #914: 5' GAA GGA TCC ATA CAT ATG AG 3' with the following conditions: 94°C for 45 sec, 55°C for 30 sec and 72°C for 90 sec for 34 cycles. The 750-bp product which included the ribosome binding site: GGA TCC ATA CAT was digested with *Bam*HI and *Eco*RI and cloned into pBluescript KS$^-$ (Stratagene) to create pGB4. This would insure a constant supply of the insert if needed, without having to amplify the fragment again. Since the bluescript clone could be liberated with *Bam*HI and *Eco*RI, it served as a further control that the product was correct. The BamHI/*Eco*RI insert was removed and cloned into the broad-host-range plasmid pBBR1MCS-2 (a gift from Dr. Michael Kovach) to create the promoterless GFP expression vector, pGB5. Although no promoter was present, the plasmid did contain a partial ribosome-binding site: GGA TCC ATA CAT, which immediately precedes the initiator methionine of GFP. This would insure that clones whose captured *cis*-acting sequences lacked such a sequence may still be able to translate GFP.

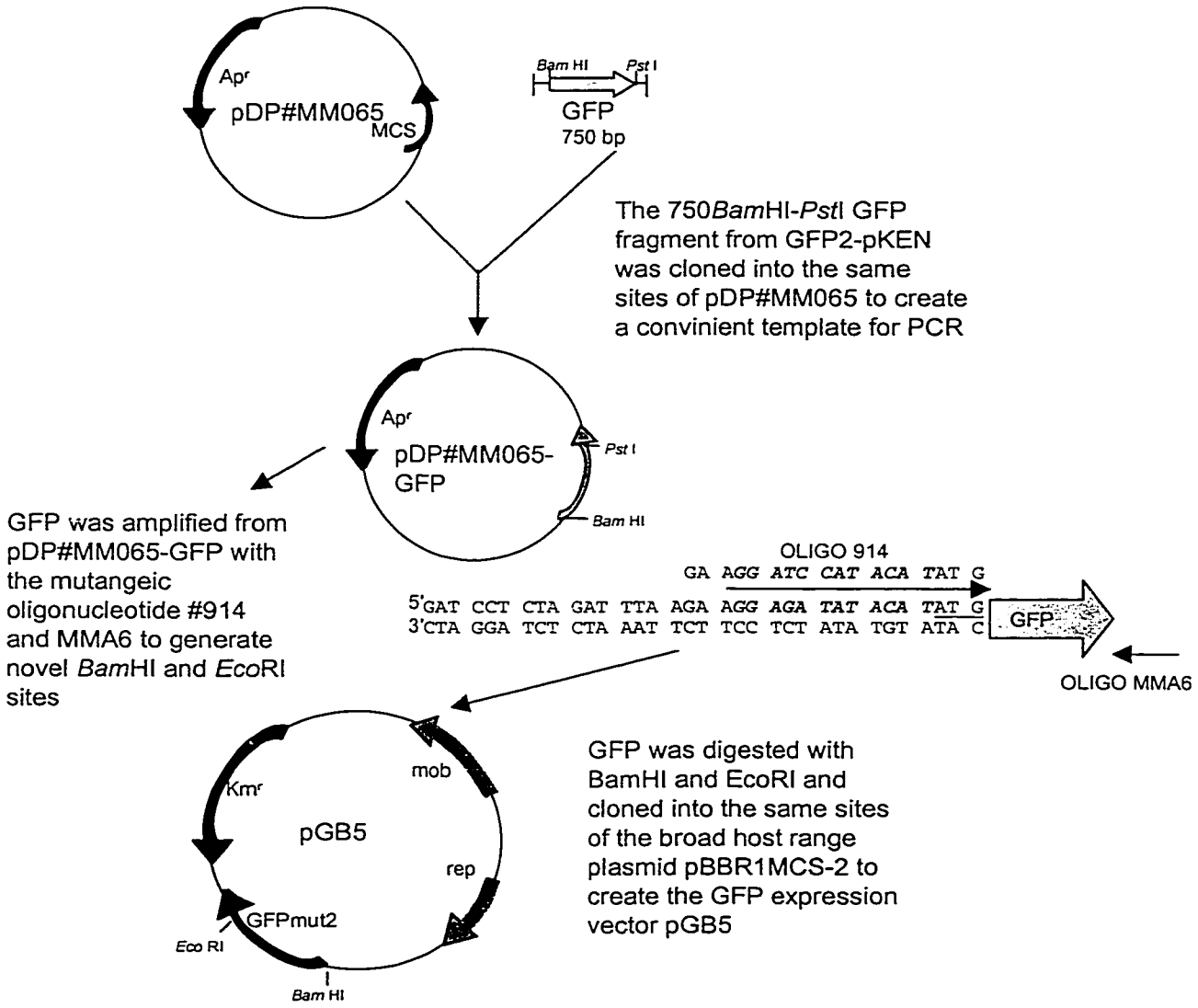


Figure 2. Construction of plasmid pGB5. The construction of pGB5, the promoterless GFP expression plasmid is diagrammed. The 750bp GFP mut2 fragment was taken from GFP2-pKEN and amplified using mutagenic oligos to create a GFP fragment with upstream ribosome binding site (bold-italics) that could be cloned as a *Bam* HI *Eco*RI fragment in the broad host plasmid, pBBR1MCS-2.

The 364-bp *vrg6* promoter sequence was amplified from 1 μg *B. pertussis* chromosomal DNA with the following oligonucleotides: #912 5' CGG GCA TGG ATC CTC GTG 3' and #913 5' CCC GGA TCC CAC ACG GGC CAG 3' which anneal at position 24 and 388 of the published sequence (Beattie *et al.*, 1992). This resulted in a *vrg6* promoter with novel flanking *Bam*HI sites, which were used to clone into the *Bam*HI site of pBluescript KS^- to create pGB3. The *Bam*HI-*vrg*6 insert from pGB3 was then cloned into pGB5 resulting in plasmids with the promoter in two possible orientations (pGB5-6 has a colinear fragment and in pGB5-3 it is inverted). Only clones containing the *vrg*6 promoter in proper context were productive fusions able to express GFP. The strategy is outlined in Figure 3.

2.4 Preparation of B. pertussis samples for electrophoresis.

BP338 (pGB5, pGB5-3, pGB5-6 and pGB5-P1) cultures were grown in SSB +/- modulators for three days. All cultures grew to approximately the same density (A_{540} 0.9) and were adjusted to 4.5 by centrifugation and suspended in SDS-gel loading buffer prior to sonication in ice-cold water for 2 minutes. Cultures were subsequently boiled for 5 minutes to further lyse bacteria prior to electrophoresis.

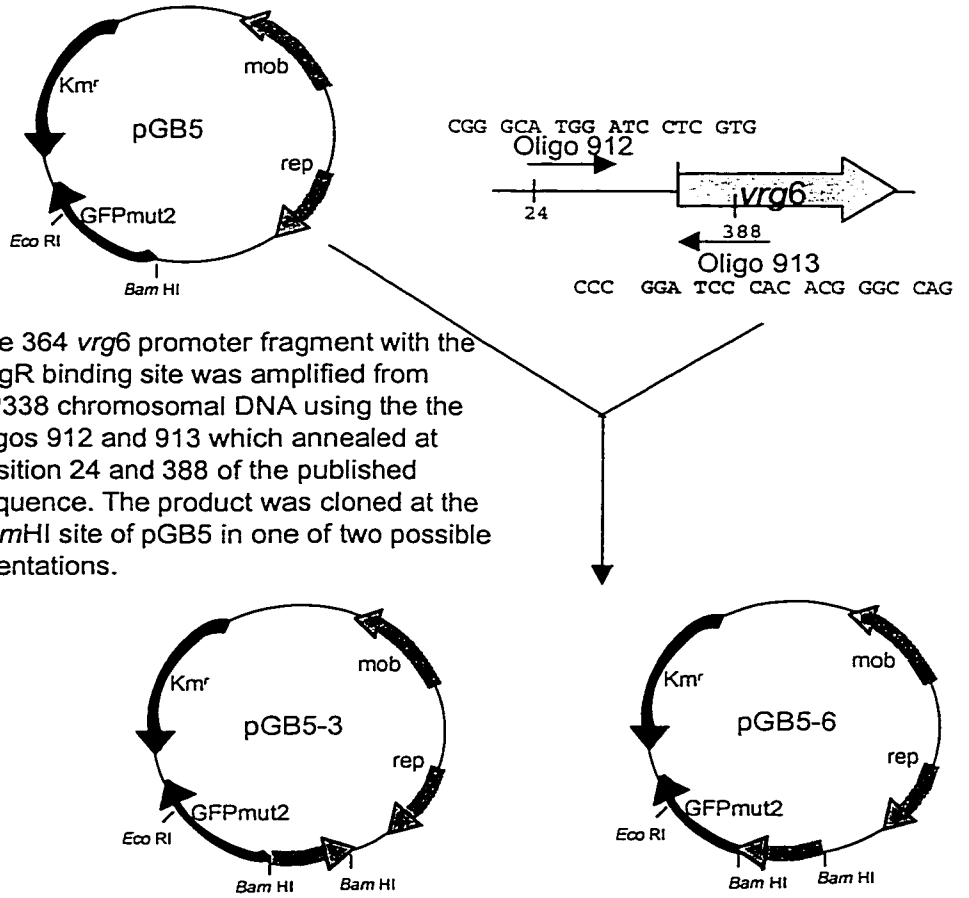


Figure 3. Construction of pGB5-3 and pGB5-6. The construction of pGB5-3 and pGB5-6, the vrg6 reporter fusions is diagrammed.

2.5 SDS-Page and Western Blot

Samples were analyzed by electrophoresis on standard SDS-polyacrylamide gels (Maniatis, 1982). Approximately 20μg of cell lysate was added per lane and gels were run in duplicate for subsequent Coomassie Brilliant Blue staining and Western Blot transfer. Electrophoretic transfer of proteins to nitrocellulose membranes was accomplished with a Novex electroblot apparatus according to the method of Towbin *et al.* (1979). A Novex electroblot apparatus was used in accordance with manufacturer's instructions: 1 hour transfer at room temperature at >250 mamps. or 25 V. Following electrophoretic transfer the blot was incubated overnight at 4°C in Blotto (100mM Tris-HCl pH 7.5, 1% tween-20, (T-TBS) and 5% skim milk powder). Blots were washed once in T-TBS and further incubated at room temperature for 1-2 hours with α-GFP rabbit polyclonal sera (Molecular Probes) diluted 1/1000 in Blotto. Blots were washed 3-5X with T-TBS and incubated with an alkaline phosphatase conjugated secondary antibody (anti-rabbit alk phos) diluted 1/5000 in T-TBS for 2 hours. Blots were further washed 5X and developed according to standard protocols with NBT/BCIP (Maniatis, 1982) until the color development reached the desired intensity.

2.6 Creation of BP338 genomic library.

A *B. pertussis* library of *Sau*3AI partially digested products was created in pGB5. This enzyme was chosen since its four-base recognition sequence, 5'-

GATC-3', occurs approximately once every 256 base pairs in a genome of 50% GC content. Partial fragments can be ligated at the *Bam*HI site of the expression vector. 100 μg of BP338 chromosomal DNA were digested with 1μl *Sau*3AI for five minutes at 21^{0}C to achieve partial digestion. The sample was visualized by agarose gel electrophoresis and digested fragments between 500 bp and 2 kpb were further purified by extraction from low-melt agarose according to standard methods. 320 ng of purified fragments were ligated to 40ng of *Bam*HI digested and SAP treated pGB5 in a volume of 50μl at 4°C for 48-96 hours. The SAP (shrimp alkaline phosphatase) treatment insured that less vector would self-ligate,thereby increasing the cloning efficiency and lowering the background. Following ligation the samples were prepared for electroporation into *B. pertussis* by removal of residual salt as follows. Carrier tRNA (10μg) was used to precipitate the DNA in the presence of 2 volumes 95% EtOH and ¼ volume 10M ammonium acetate following centrifugation. The pelleted DNA was resuspended in 10μl double distilled H_2O (dd H_2O) and used to transform 2 x 500 μl aliquots of electrocompetent BP338.

2.7 Electroporation of *Bordetella pertussis*

Electrocompetent BP338 was made following the procedure of J. F. Miller, Stanford University (unpublished data), with modifications. Briefly, BP338 was grown in 1L Stainer-Scholte broth for 3 days in a 2-L Fernbach flask with moderate shaking at 100 rpm at 37°C. Cells were pelleted by centrifugation at

4000 rpm, washed twice in 50 ml ddH_20, resuspended in 10 ml electroporation medium (272 mM sucrose and 15% glycerol) flash frozen in liquid nitrogen and stored at –80°C. When needed, cells were thawed on ice and 500 µl aliquots were added to cold electroporation cuvettes (0.2 cm electrode gap). Up to 5 µl of a purified ligation reaction was added to the cells and allowed to equilibrate on ice for 15 minutes. Cells were pulsed using a BioRad pulse controller (Richmond, Ca) set at 400 ohms, 2.5kV and 25µF and immediately outgrown at 37°C in 1 ml Stainer-Scholte broth for 2-3 hours. Cells were centrifuged at 5,000 rpm for 10 min and plated onto selective BGA plates (Nal, Kan, Mod). Small (0.5 mm) colonies typically developed in three to five days.

2.8 Screen for *bvg*-repressed promoters in *Bordetella pertussis*

The GFP expression library was screened for modulation-specific promoters, in transformed *B. pertussis,* by observing colonies under a Leica Instruments microscope equipped with a mercury lamp and FITC filter. GFP-expressing bacteria appeared as fluorescent apple-green colonies and were readily distinguished from non-expressing pale yellow colonies (Figure 4). Colonies expressing GFP in the presence of modulators were re-tested by streaking onto BG plates (Nal, Kan) with and without modulators and allowed to grow for 3 days. Fluorescence was subsequently measured by flow cytometry. To prepare samples for cytometry, agar-grown *B. pertussis* were suspended in 2 ml of 50 mM Tris-63 mM glutamate-43 mM saline buffer (TGS, pH 7.4) to an

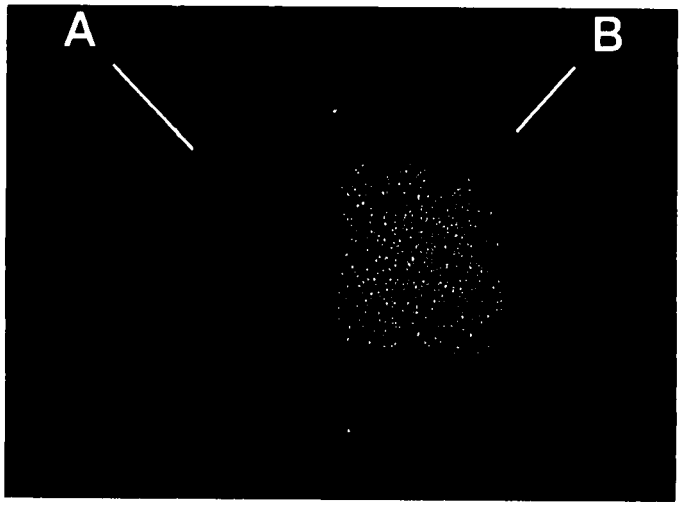


Figure 4A. Fluorescent and non-fluorescent colonies. GFP expressing colonies appeared apple-green under a dissecting microscope equipped with fluorescence and a FITC filter. Non-GFP expressing colonies had faint yellow auto-fluorescence which was readily distinguishable.Colonies were typically 2mm in diameter at the time of analysis. Shown here are two colonies that grew together on BGA-mod. The colony on the left (A) is transformed with pGB5-3, the one on the right (B), with pGB5-6

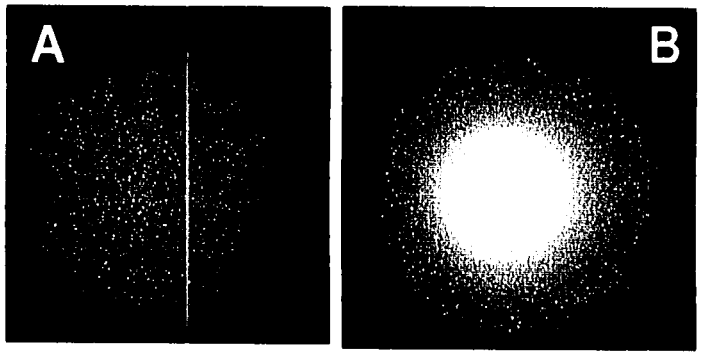


Figure 4B. Varying fluorescence intensities.Colonies with varying intensities were found during the screen. Those that were highly fluorescent, like pGB5-P1 (B) were invariably not modulation-specific. Those with diminished fluorescence on agar, like pGB5-P9 (A), had a higher likelihood of being modulation-specific.

optical density of 0.12 at 540 nm. Broth-grown cultures were suspended 1:1 in TGS (2 ml total) before analysis. All samples were analyzed immediately following suspension and were shielded from light to diminish quenching of fluorescence.

2.9 FACScan settings and calibration

All flow cytometry was performed using a FACScan cytometer (Beckton, Dickinson) and analyzed using CellQuest analysis software. Instrument settings for forward and side scatter (FSC, SSC) were determined by the size of *B. pertussis* and remained unchanged throughout the experiments at FSC: E-1 and SSC: 178V. In order to calibrate the machine, BP338 (non-modulated) and BP347 (modulated) were scanned for comparative purposes to insure that there was no detectable size differences between the bacteria. As shown in Figure 5, no detectable difference in FSC or SSC is evident between the two strains. Thus, no special parameter adjustments needed to be made before subsequent analysis.

The FITC (FL-1) channel was used to visualize GFP expression and the voltage was determined by calibrating 3.0 μm Rainbow beads of 8 varying intensities (Spherotech, Libertyville, IL), before every experiment to ensure that a predetermined peak would always exhibit the same mean fluorescence of 540 Units. To achieve this constant mean fluorescence the voltage ranged from 740-770V. During each acquisition, 5000 events based on size (FSC and SSC) were

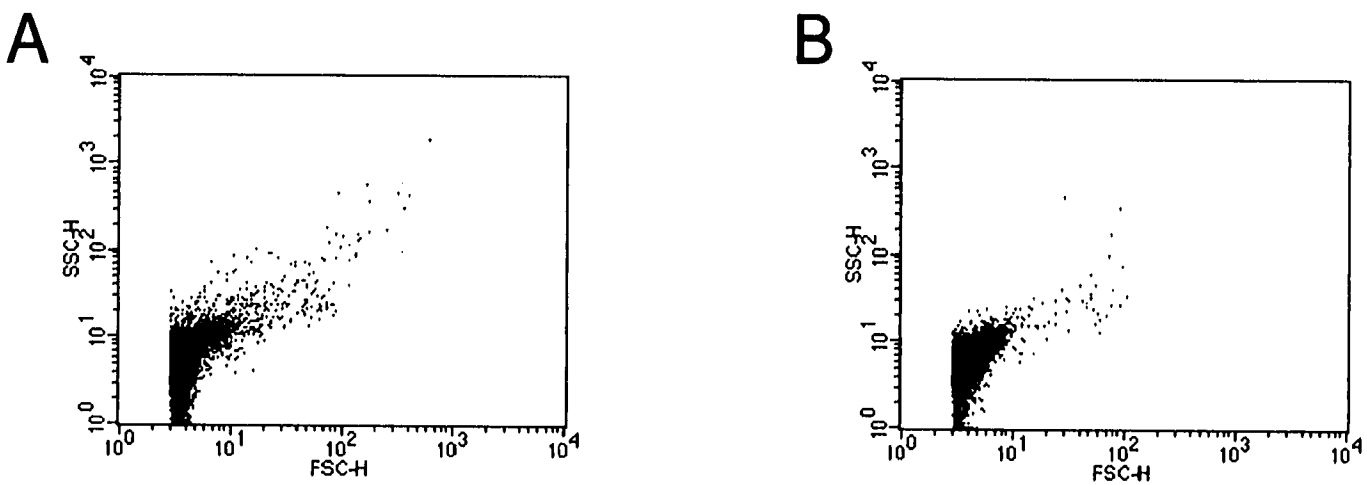


Figure 5: Forward and side scatter of broth-grown (SSB) BP347 (A) and BP338(B). Forward scatter (FSC) and side scatter (SSC) of *B. pertussis* strains was measured by the flow cytometer and used to judge the size of events being analyzed for green fluorescence. As shown in the histograms above, there is no difference in SSC *vs.* FSC between "modulated" (BP347, A) or "non-modulated" (BP338, B) bacteria.

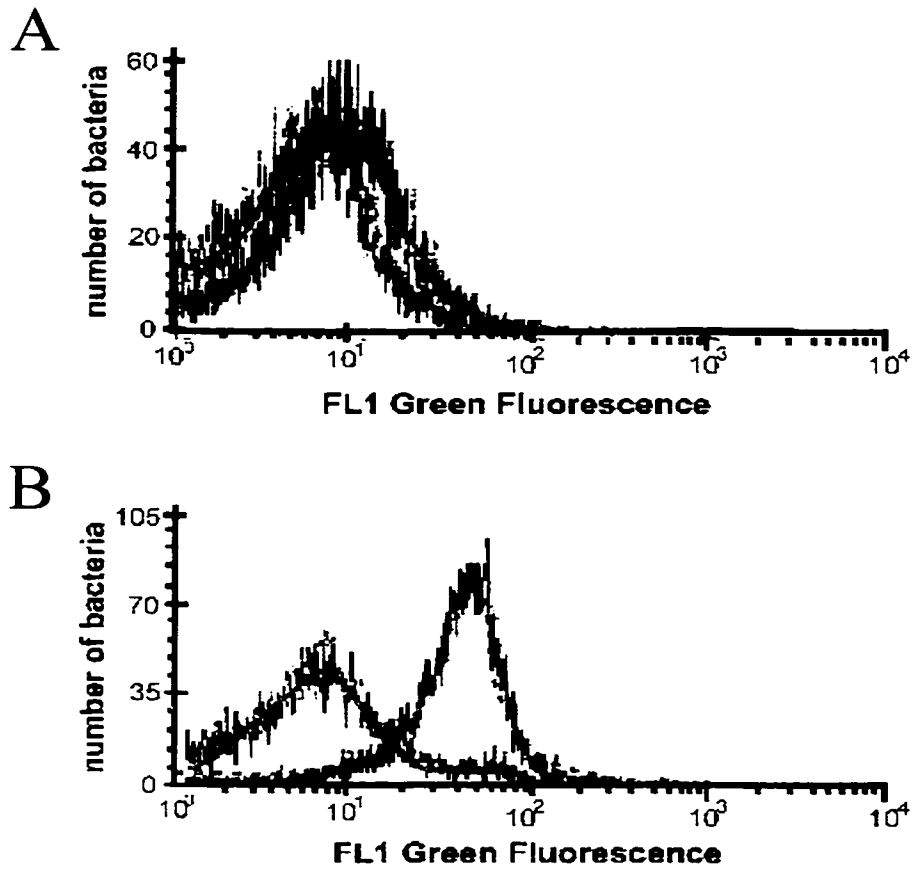


Figure 6: FL-1 histogram for modulated and non-modulated BP338(pGB5-3) (A) and BP338 pGB5-6)(B). Fl-1 fluorescence (FITC channel) was measured by FACS analysis of 5000 bacteria grown in SSB in the presence of modulators(dashed lines) and absence of modulators (solid lines). As shown, strain BP338 (pGB5-3) (A) had only background fluorescence when grown under experimental conditions, but strain BP338 (pGB5-6) showed FL-1 fluorescence when grown in the presence of modulators

analyzed. For FACS acquisition, 5000 agar or broth-grown bacteria were scanned and data collected for fluorescence intensity. Fluorescence FI-1 histograms, such as that shown in Figure 6, were collected for each clone. As was typical of every clone isolated, the bacteria with background endogenous fluorescence (below 10) had broad acquisition peaks, whereas those expressing GFP had sharp peaks (see Figure 6B for an example). Each clone was scanned in triplicate and 3 data points representing the mean of 5000 bacteria were further averaged. Data are presented for GFP induction kinetics as the mean +/- SEM.

2.10 Sequence analysis

Automated sequencing was done by the DNA core facility using an automated ABI sequencer (Department of Biochemistry, University of Alberta, Edmonton, Alberta). Manual sequencing was performed using a Thermo Sequenase kit, according to manufacturer's instructions. When possible, promoter templates for sequencing were amplified by PCR using transformed *B. pertussis* colonies or purified plasmids. Plasmids were released from cells for colony PCR by repeated freezing and boiling of a single colony suspended in 50 μl sterile ddH20. 5 μl of the lysed colony suspension was used as a template for standard PCR reactions. Primers used for amplification of the promoter DNA were designed to bind flanking plasmid sequences PGB5 PCR: 5'GCT GCA AGG CGA TTA AGT TGG 3' and PGFP: 5'CAA GTG TTG GCC ATG GAA CAG

G 3'. Internal primers were used individually for manual sequencing following PCR product purification: PGB5 SEQ: GTA ATA CGA CTC ACT ATC GG 3' and 5' GTG CCC ATT AAC ATC ACC ATC 3'. When plasmid templates were used PGFP SEQ and PGB5 SEQ served as primers for sequencing. In both cases, PGFP SEQ was used initially for sequencing unknown fragments and PGB5 SEQ was used subsequently for sequencing small promoters. A standard BlastX search was used to determine the identity or homology of the *B. pertussis* promoters.

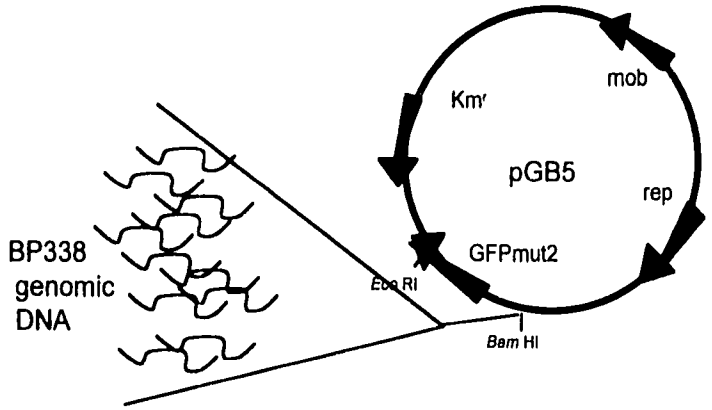
3. Results

3.1 Identification of *Bordetella pertussis* modulation-specific promoters (msps).

Modulation of *B. pertussis* in response to environmental signals results in the simultaneous down-regulation of classical virulence factors (toxins, adhesins) and the de-repression of *bvg*-repressed genes, resulting in their expression. (Lacey 1960, Uhl and Miller 1995). In order to elucidate the role of modulation in the lifecycle of *B. pertussis*, an open-ended "promoter–trapping" screen was developed to discover genes that are up regulated in response to modulating signals (Figure 7). The modulated state can be induced by growth on BGA in the presence of $MgSO_4$ (20 mM) and nicotinic acid (5 mM) (Melton and Weiss 1993, Lacey 1960). This suggested that a screen for modulation-specific promoters could be performed by cloning random DNA fragments upstream of a

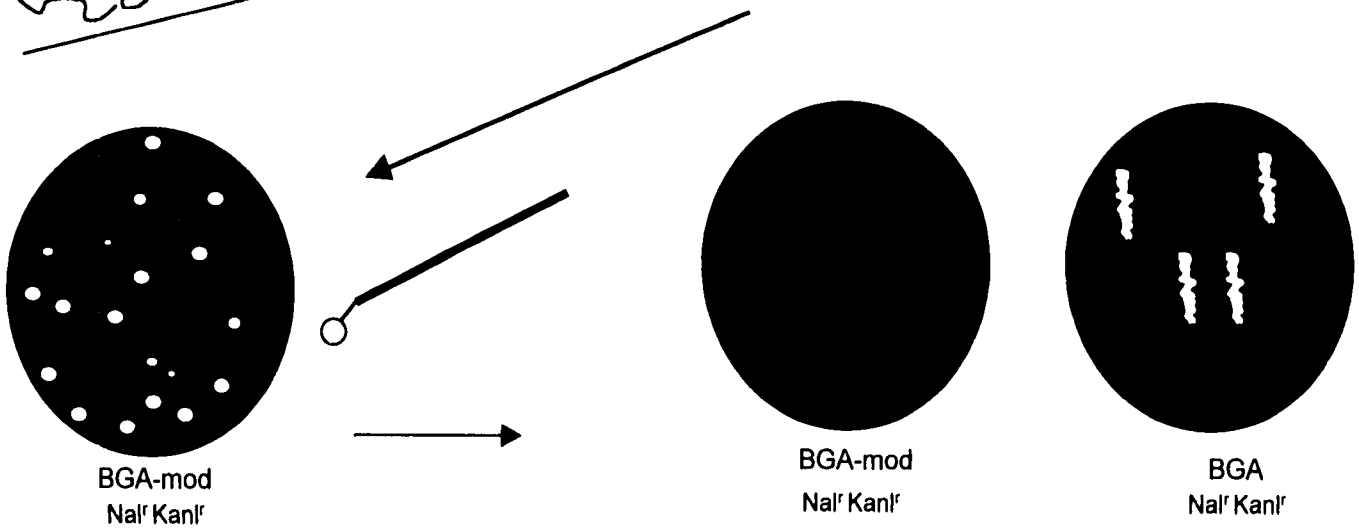
Figure 7. DFI selection strategy for *bvg*-repressed promoters.

1. BP338 *Sau* 3AI partial size-selected DNA fragments were ligated to pGB5 to create the GFP expression library



2. The library was electroporated into BP338

3. Transformants were selected by colony microscopy for GFP expression under modulating condition.



4. Fluorescent colonies were re-streaked and those that expressed only under modulating conditions were chosen for subsequent testing by flow cytometry

Figure 8. BvgR Binding site and consensus. The BvgR binding site has been found in 4 of 5 *bvg*-repressed genes previously described. It is typically 10-30 bp after the start of transcription.

vrg 6	GTTGCTGCCGGCATCGGCGCTGCCGGACTCAT
vrg 18	GCTGTTGCCTGCCGCGGCGCTGCTGGGCGGCT
vrg 24	ATGCTTGCCGGCCTGGCCGCAGTGATGCCCAT
vrg 53	GTCGCCGCCGGCAGTGCTGCTGCCCTGCCCAT
consensus	GT.G.TGCCGGC...G.CGCTGC...GC.CAT

heterologous reporter, Green Fluorescent Protein (GFP). It was shown previously that a downstream *cis*-acting sequence found in four of the five *bvg*-repressed genes discovered previously was sufficient to confer BvgR binding and BvgAS modulation-specific control (Figure 8; Beattie *et al.*, 1993). It was unknown, however, if GFP could be properly expressed and folded in *B. pertussis*. In order to determine if such an approach was likely to be successful, a control *vrg6::gfp* fusion with an intact BvgR binding site was constructed (pGB5-6) and tested for modulation-specific expression of GFP by fluorescence microscopy. The identical plasmid containing the same promoter in an inverted orientation (pGB5-3) was used as a negative control (Figure 3). As shown in Figure 4A only pGB5-6 had an active GFP fusion, capable of fluorescing under UV light while pGB5-3 showed no such fluorescence. Bacteria harboring either pGB5-3 or pGB5-6 appeared as regular pale yellow colonies when grown under non-modulating conditions (see Figures 10A and 11A for numerical data). GFP fusion-protein levels were further corroborated by SDS-PAGE of BP338 followed by α-GFP Western Blot analysis (Figure 9A and 9B). Of the control strains, pGB5, pGB5-3 and pGB5-6 only pGB5-6 with a collinear *vrg6* promoter fusion expressed GFP when grown on BGA with nicotinic acid and sulfate. This further substantiated that clone pGB5-6 encoded a productive GFP fusion and moreover, it was under modulatory control. In addition, this confirmed that heterologous GFP expression was possible in the fastidious *B. pertussis*, and that a screen for novel modulation-specific promoters was likely to yield candidate genes. A constitutive promoter fusion, pGB5-P1, an RNA polymerase::GFP fusion is

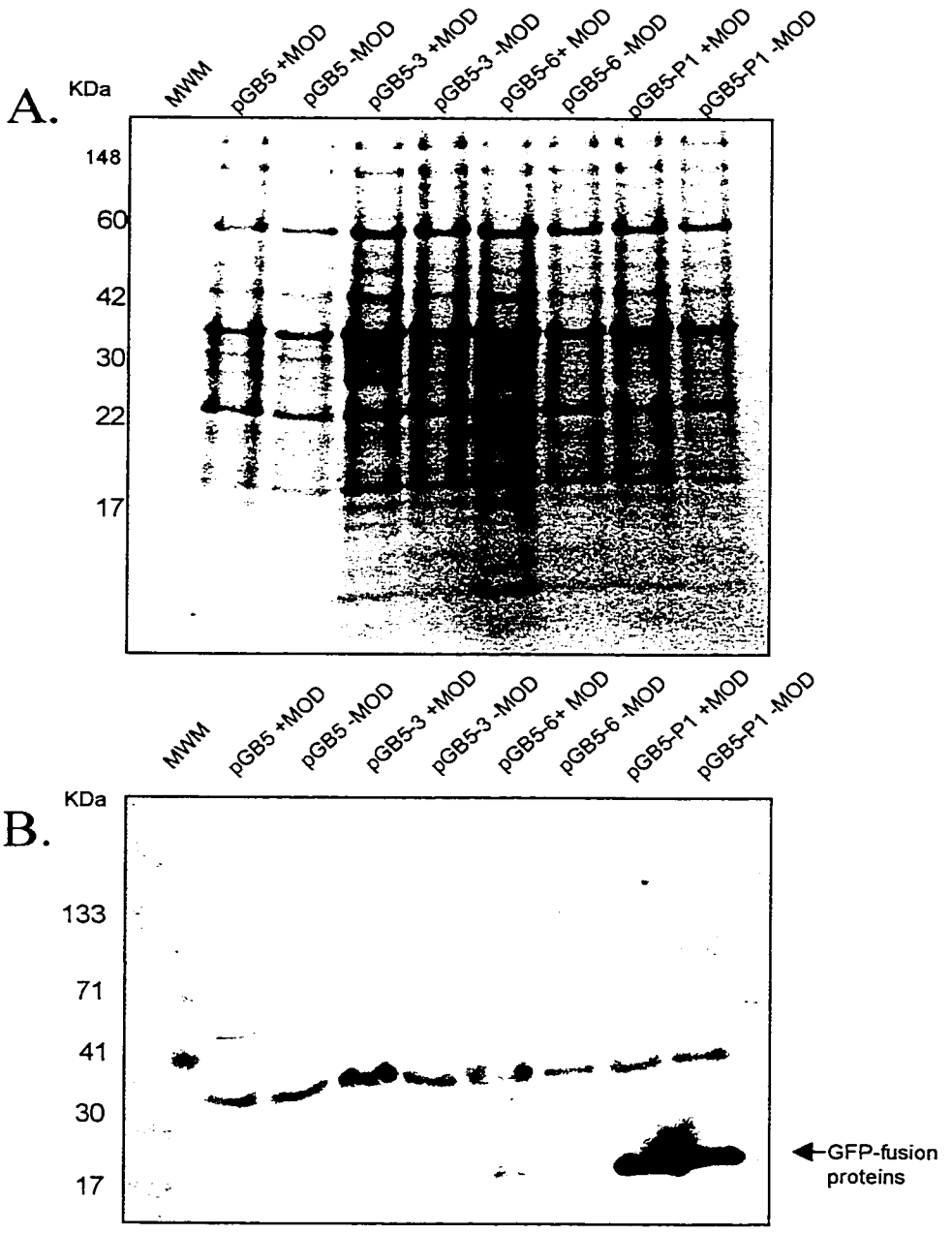


Figure 9A,B: SDS-Page(A) and Western Blot (B) for BP338 control strains. Strains were grown for three days in SSB with and without modulators(+mod and -mod) to A_{540} 0.9 and harvested for SDS-Page and Western Blot . Bacteria were lysed in SDS-loading buffer, boiled for 5 minutes and briefly sonicated. 20μg protein was loaded per gel lane. Gels were stained with Coomassie Brilliant Blue. Blots were probed with an α-GFP polyclonal antibody (Molecular Probes) followed by an alkaline phosphatase conjugated secondary and subsequently developed with NBT/BCIP according to standard protocols.

included for comparison purposes between typical *bvg*-repressed promoters and strong constitutive ones.

A series of four promoter libraries from partially *Sau*3AI-digested BP338 (Tohama I) DNA were size-selected by standard agarose gel electrophoresis in order to obtain promoters that were between 0.5-2 kb in size. This was to insure that small fragments, which would clone preferentially, would be excluded in the library. The fragments were cloned into the GFP expression vector pGB5 and over 2000 BP338 transformants were screened. Bacteria expressing GFP on BGA appeared as apple-green colonies by fluorescence microscopy and were readily distinguished from non-expressing pale yellow colonies. 40 clones expressing GFP under modulating conditions were identified; however, 70% expressed GFP constitutively, as determined by replica streaking onto BGA with modulators and regular BGA agar, as determined by fluorescence microscopy. Interestingly, most of the constitutive clones were very brightly fluorescent on agar, whereas modulation-specific promoters were never as bright, in comparison (Figure 4B). Five modulation-specific clones representing a range of GFP intensity, as assessed by preliminary microscopy and FACS sorting, were chosen for subsequent analysis by flow cytometry (pGB5-P2, pGB5-P5, pGB5-P7, pGB5-P9 and pGB5-P10).

3.2 GFP induction kinetics and DFI of agar- and broth-grown BP338 transformants

In order to ascertain the dynamics of GFP expression, agar-grown clones were subjected to FACS analysis over a three-day period. Since *B. pertussis* colonies take at least two days to mature, it was necessary to analyze the colonies between day two through four of growth. Induction kinetics for agar-grown bacteria and control strains are presented in Figures 10-17(A). Clones were analyzed in triplicate over the three-day time course and each graphed point represents the mean of three independent experiments. Approximately $5x10^3$ individual bacteria were scanned in each separate experiment to generate one mean data point. All modulation-specific promoters had a range of mean fluorescence between 10 to 35 units (U, normalized to Rainbow Bead standards) when grown on agar. Although this was a low value, it was comparable to the *bvg*-repressed control strain pGB5-6 (*vrg6::gfp,* Figure 12A) which expressed a mean fluorescence between 20 to 25 U over three days. Background endogenous fluorescence (pGB5 and pGB5-3) seldom registered over 10U (Figures 9A and 10A).

Although the clones were similar in the absolute levels of GFP expressed, they differed in the temporal expression of GFP. Control strain pGB5-6 (Figure 12A) and clone pGB5-P2 (Figure 13A) maintained almost constant GFP expression on BGA with modulators over the three-day time course, whereas clones pGB5-P7, pGB5-P9 and pGB5-P10 expressed maximal GFP on day four (Figures 15A, 16A, 17A). One clone, pGB5-P5 had peak expression in the middle time point,

Figures 10-17: GFP induction kinetics for agar and broth-grown *B. pertussis* GFP expression clones. Bacterial strains with following plasmids: pGB5, pGB5-3,pGB5-6, pGB5-P2, pGB5-P5, pGB5-P7, pGB5-P9 and pGB5-P10 were harvested in triplicate from either Stainer-Scholte broth or Bordet-Gengou agar and subjected to FACS analysis over a three-day time course. The means +/- standard error of the mean (SEM) of three scans consisting of 5000 bacteria each were averaged to generate the data points shown. The three-day time course is shown for agar and broth-grown BP338 (wild-type strain) and BP907 (a BvgA$^-$ strain) in the presence and absence of modulators (+mod and -mod respectively).

Figure 10: GFP induction kinetics of agar and broth-grown *B. pertussis* (pGB5)

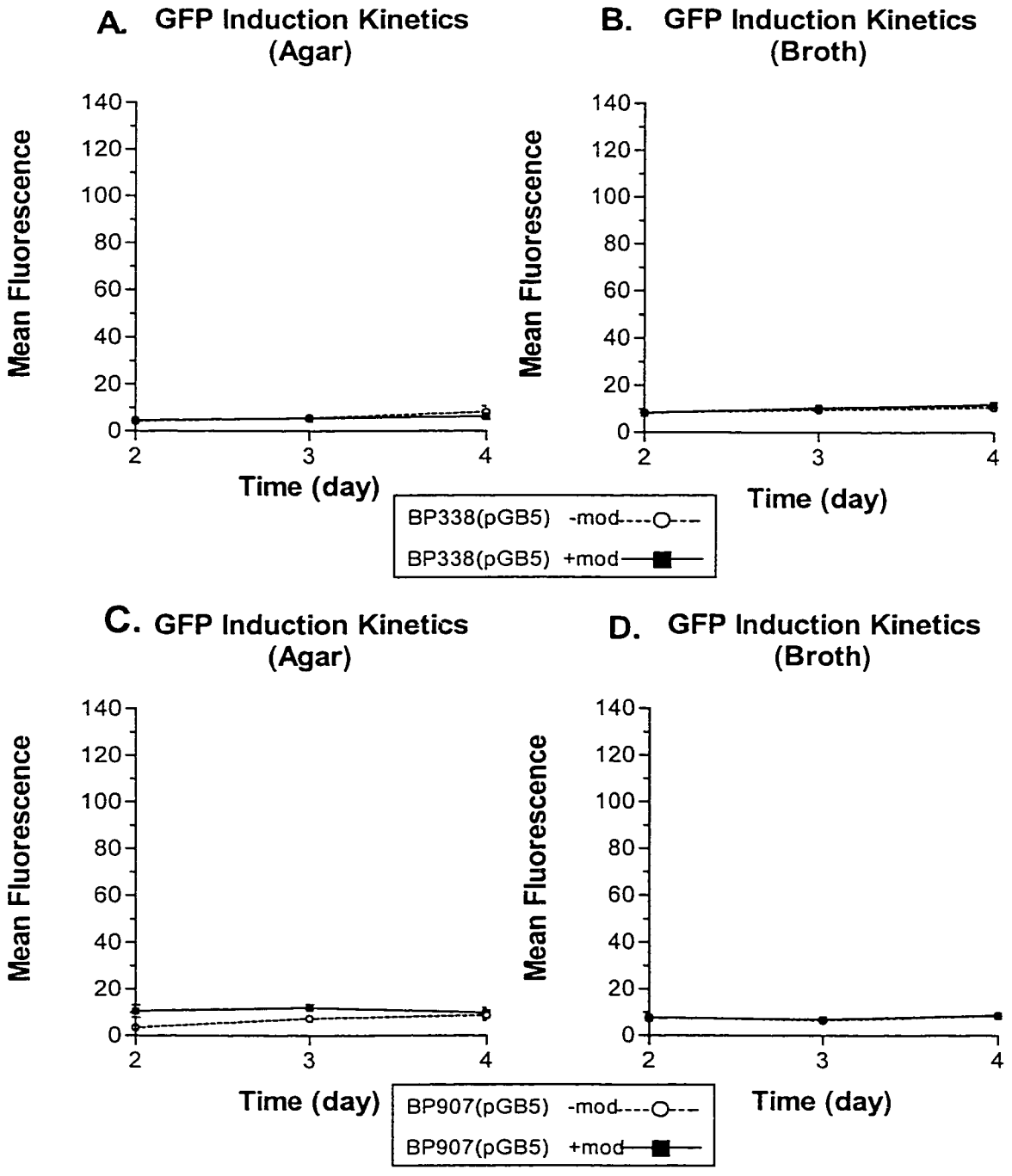


Figure 11: GFP induction kinetics of agar and broth-grown *B. pertussis* (pGB5-3)

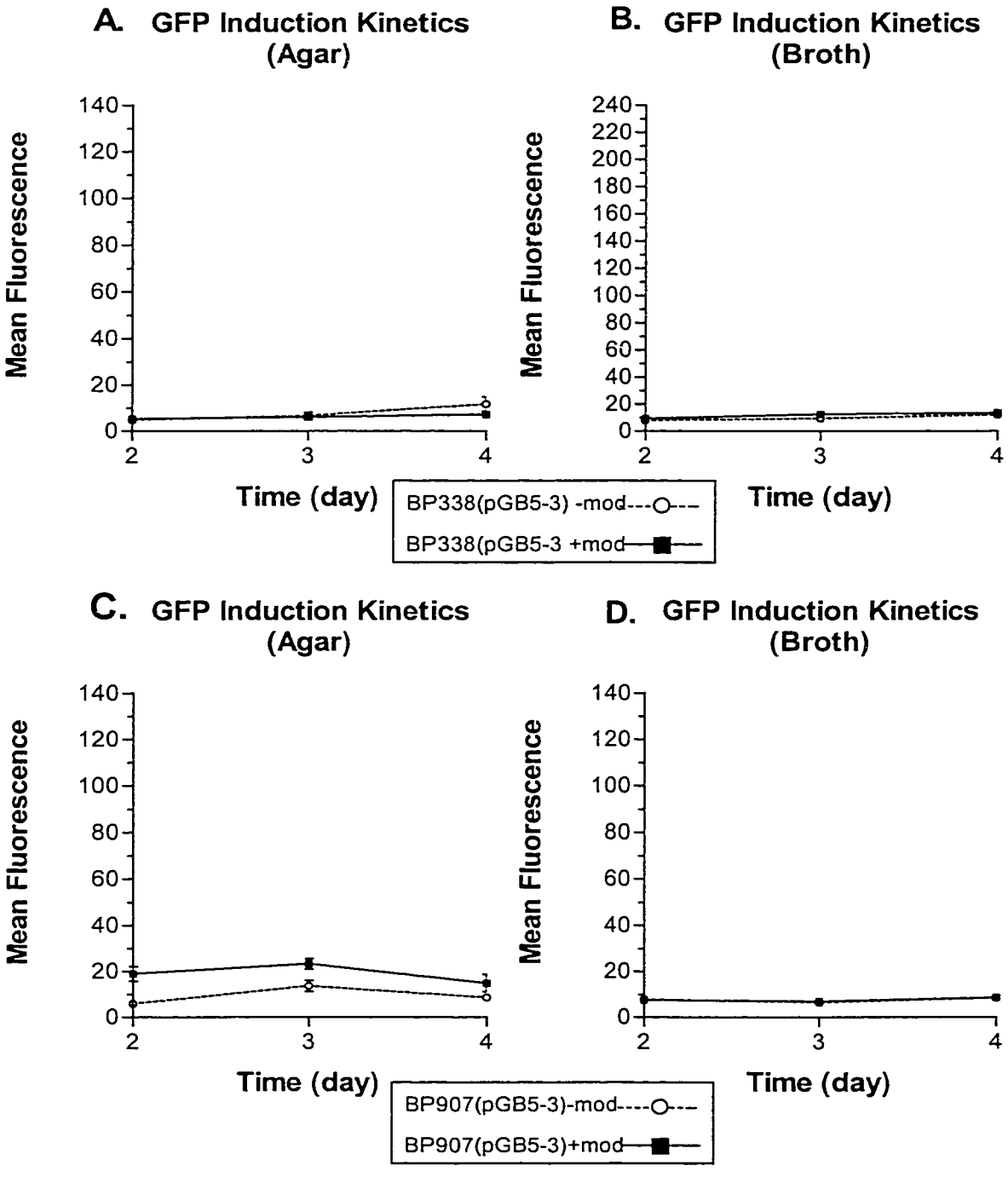


Figure 12: GFP induction kinetics of agar and broth-grown *B. pertussis* (pGB5-6)

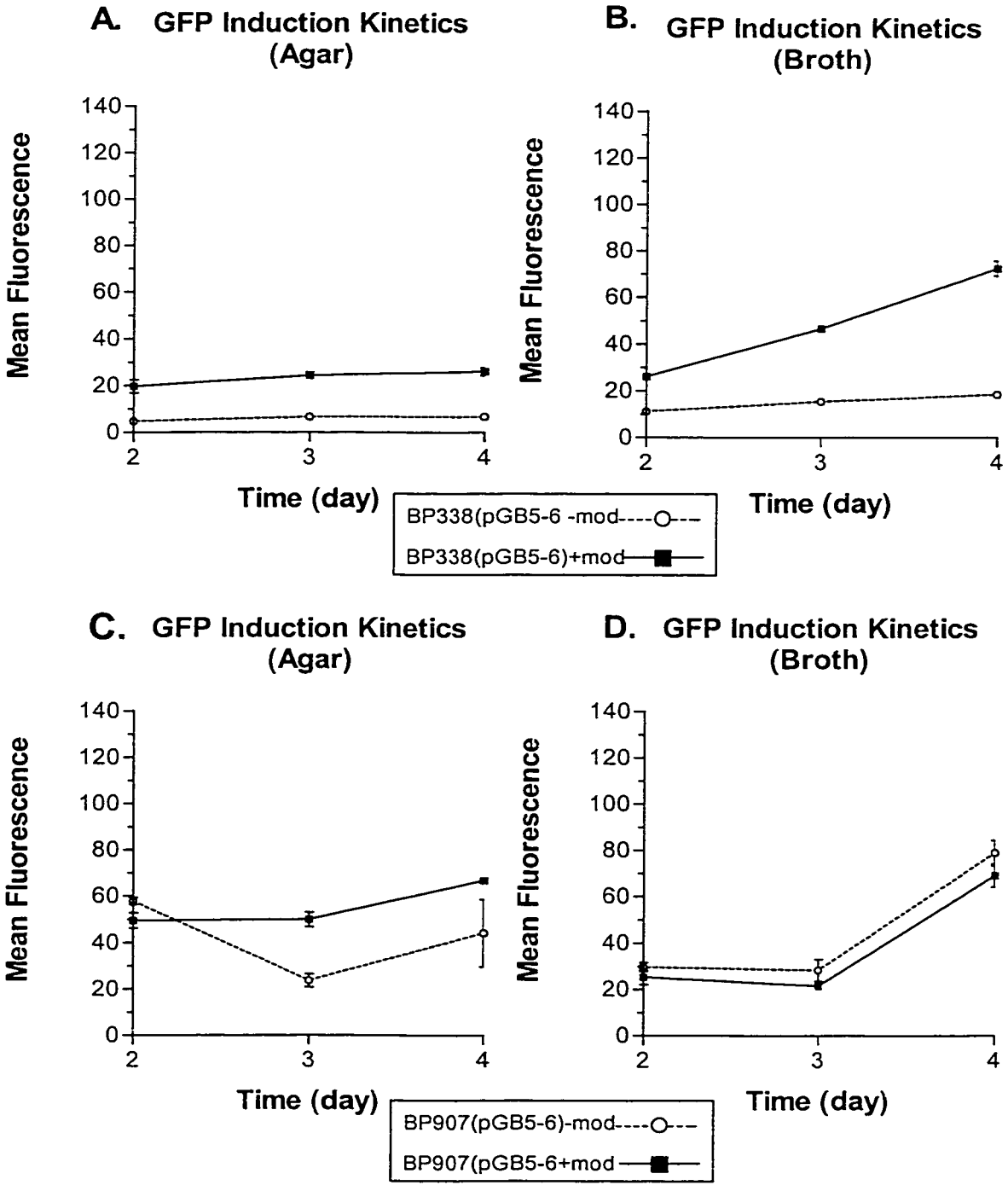


Figure 13: GFP induction kinetics of agar and broth-grown *B. pertussis* (pGB5-P2)

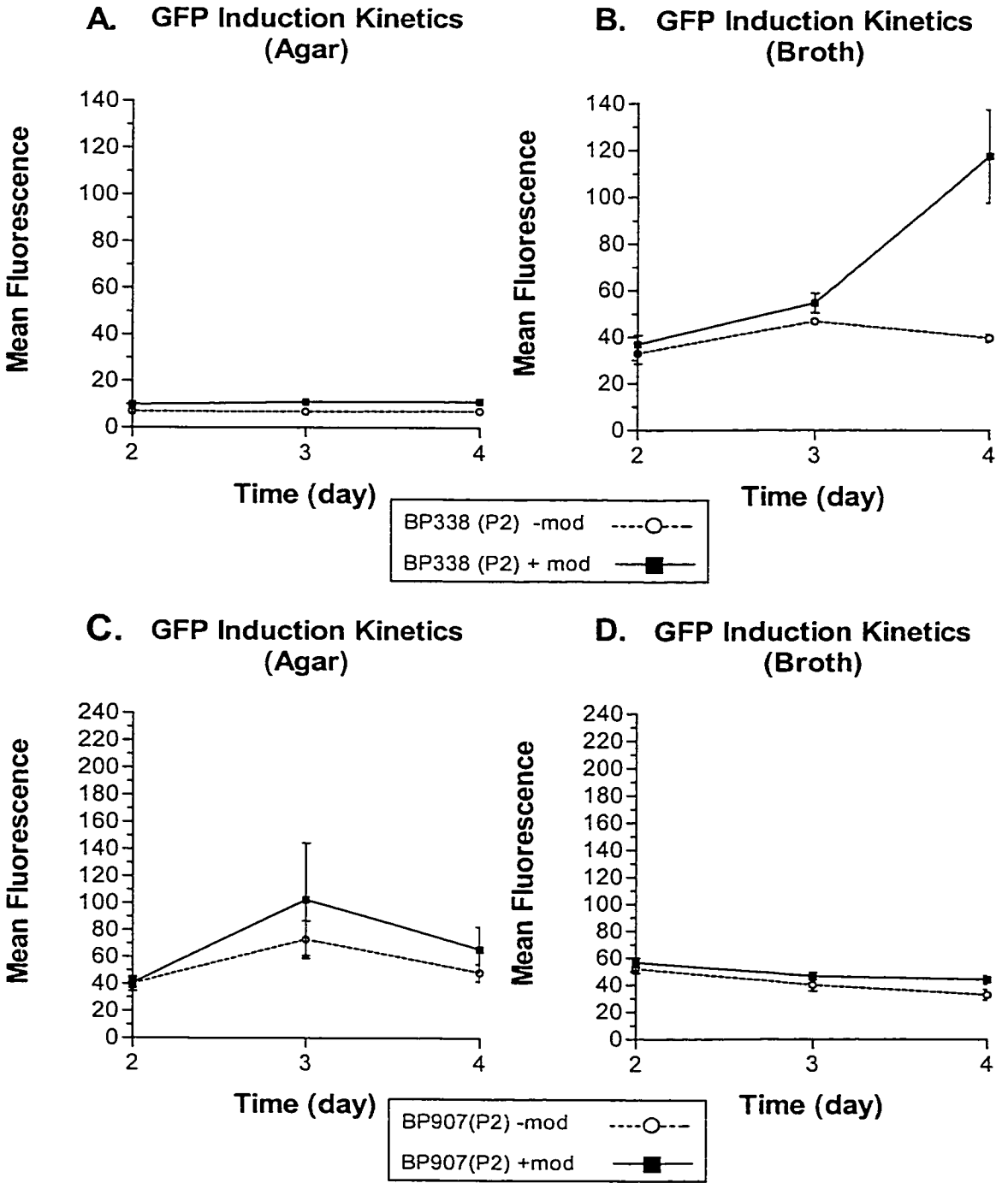


Figure 14: GFP induction kinetics of agar and broth-grown *B. pertussis* (pGB5-P5)

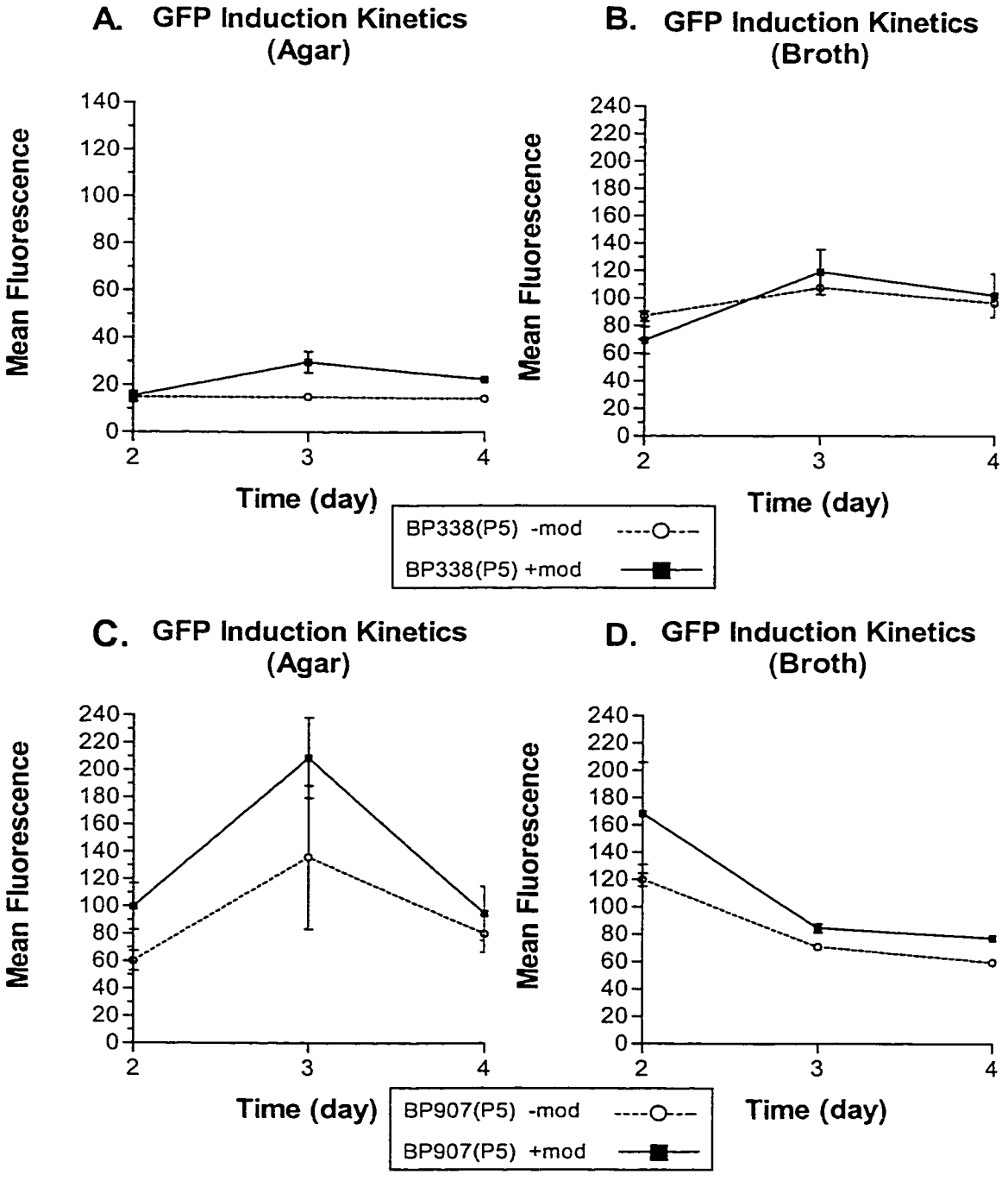


Figure 15: GFP induction kinetics of agar and broth-grown *B. pertussis* (pGB5-P7)

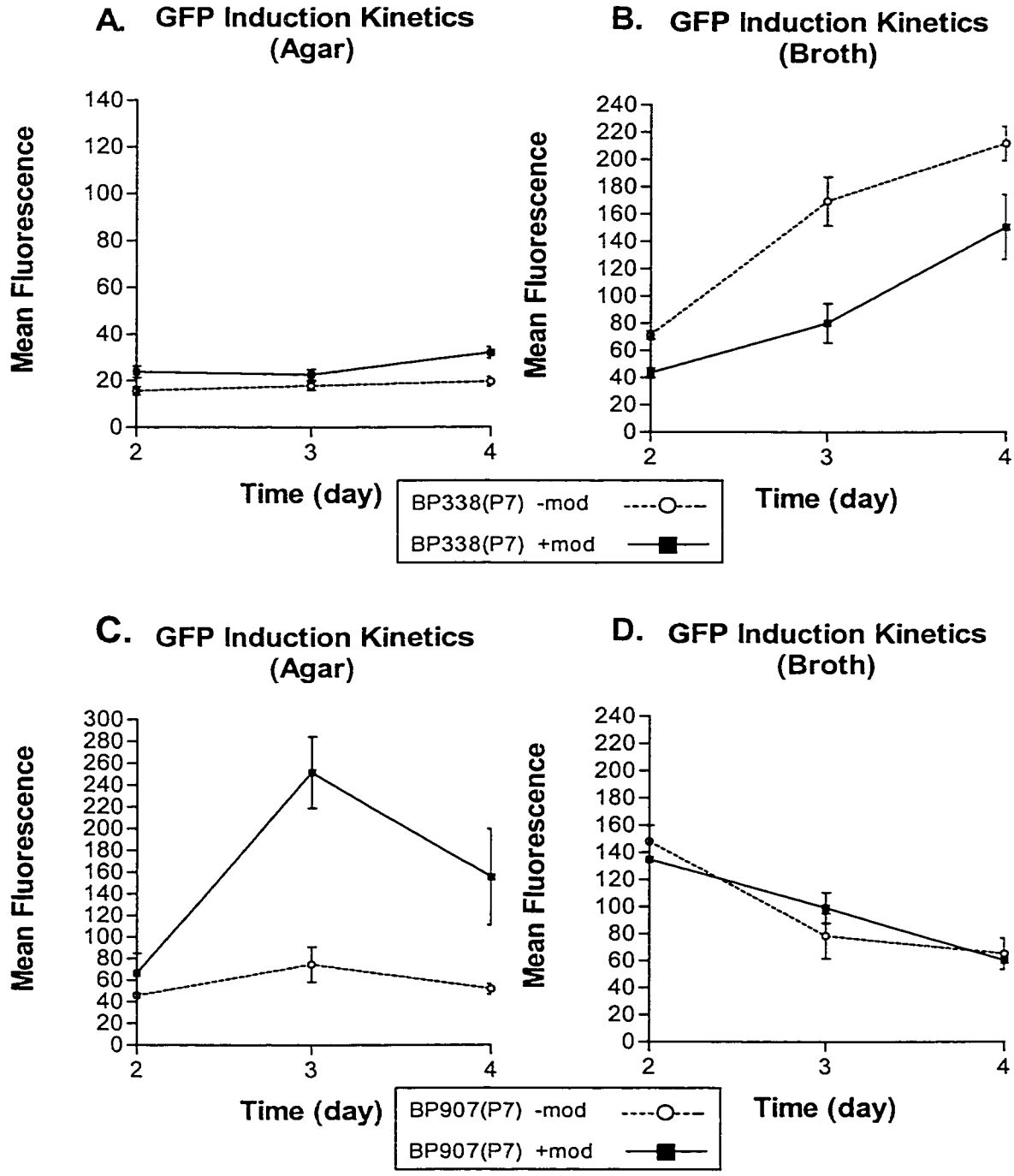


Figure 16: GFP induction kinetics of agar and broth-grown *B. pertussis* (pGB5-P9)

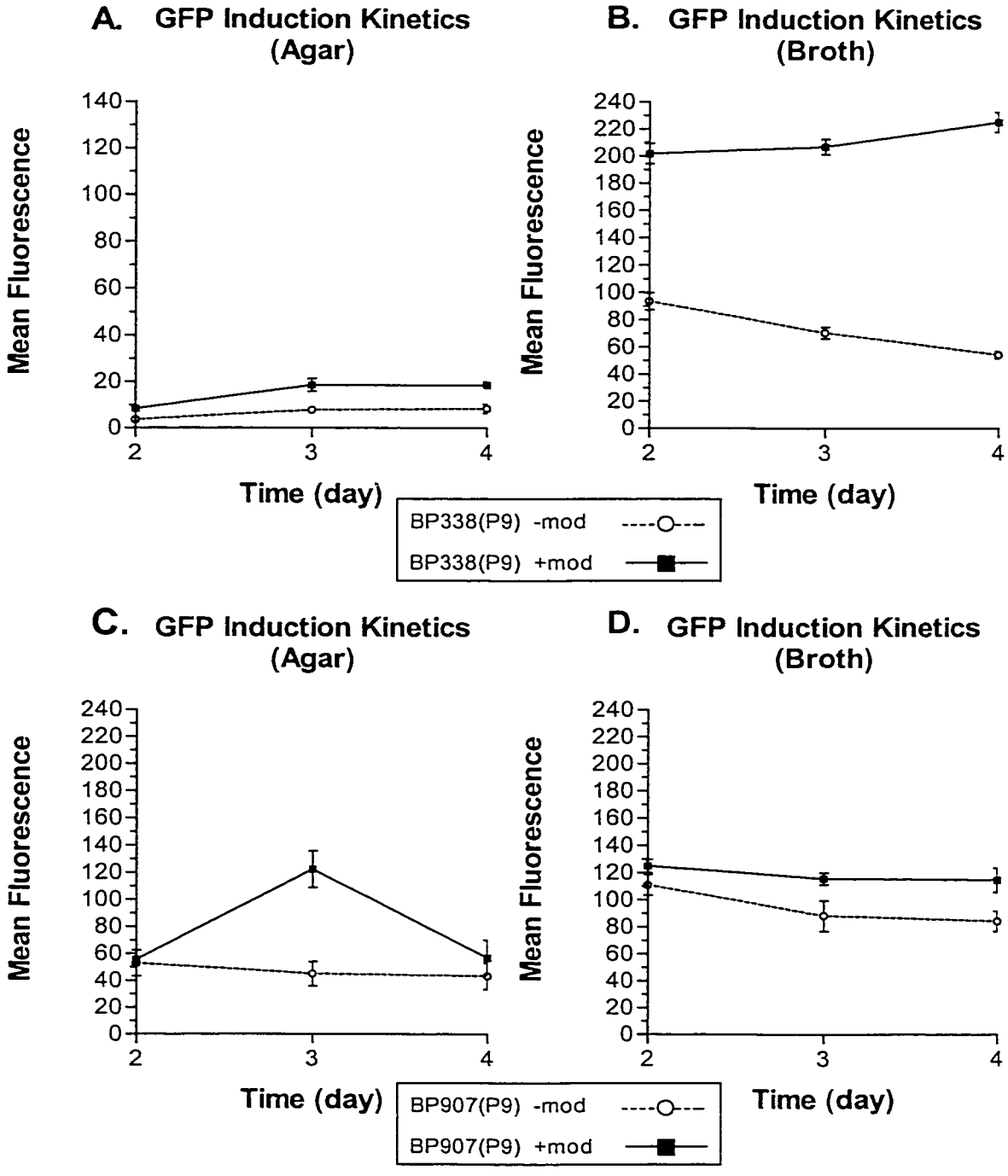
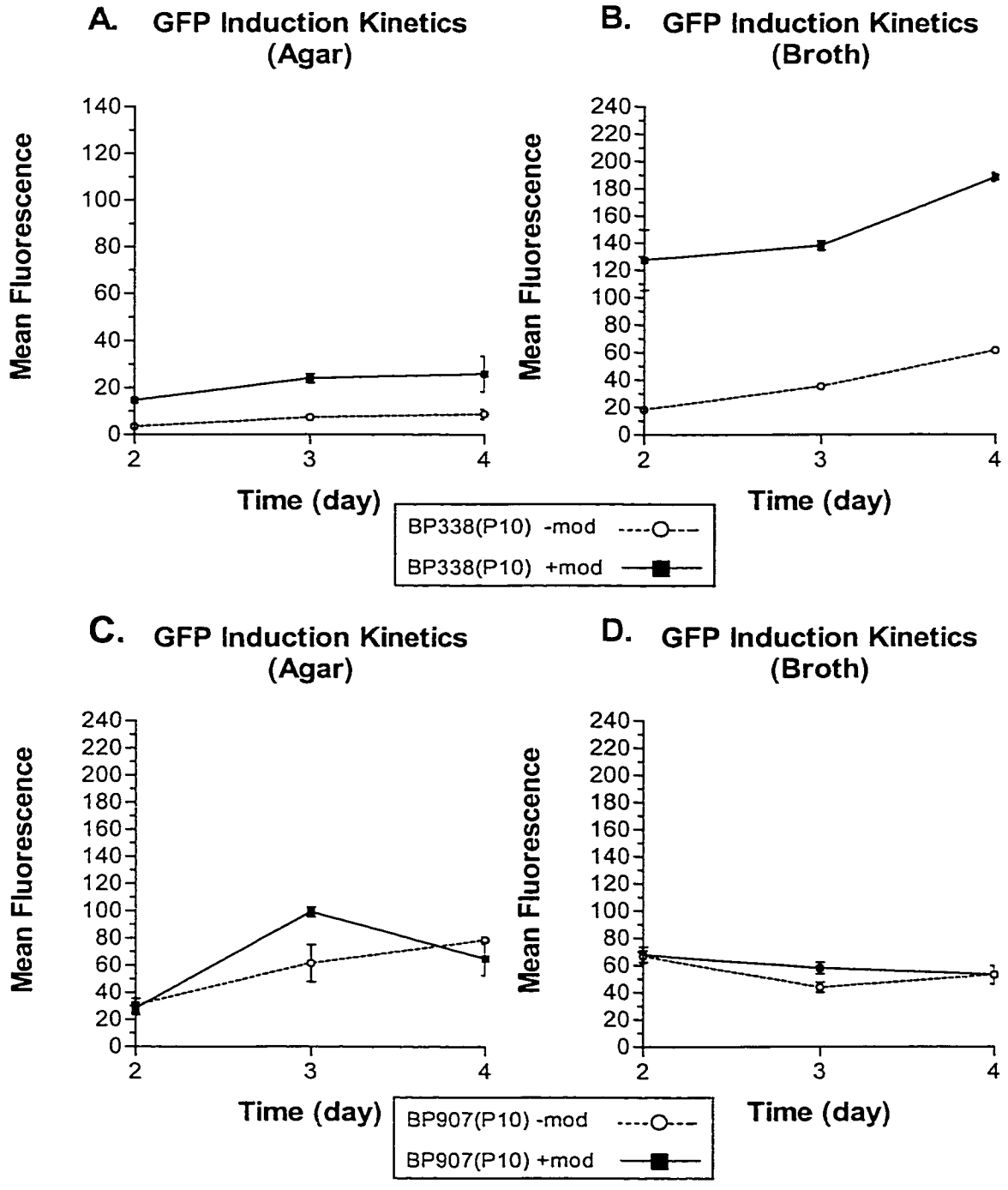


Figure 17: GFP induction kinetics of agar and broth-grown *B. pertussis* (pGB5-P10)



day three (Figure14A). Thus various clones showed temporal differences in GFP expression and could be grouped into early (pGB5-P5), constant (pGB5-6, pGB5-P2) or late (pGB5-P7, pGB5-P9, pGB5-P10) modulation-specific promoters.

Since the expression of high copy *bvg*-repressed fusions on BGA are known to be leaky, clones grown on BGA with modulators were compared to those grown on BGA with no modulators (Beattie, *et al.* 1993) (Figure 18A). The resultant ratio, called differential fluorescence induction (DFI) was used to express the fold induction of the clones grown under various conditions. As expected, the DFI for the control strains pGB5 and pGB5-3 remained essentially unchanged (Figure 18A). The clones showed modulation-specific induction, which ranged between 1.5-4.5 fold over non-modulating conditions, consistent with the prototypic *vrg*6 (pGB5-6), which had 4-fold induction. For the majority of test clones, the DFI peaked early (day two and three) or stayed essentially the same (pGB5-6, P2, P9, P5 and P10) despite the late expression of high GFP levels. This is likely the result of leaky reporter expression as the cells approach stationary phase coupled with the accumulation of GFP inside the cell. Indeed, the majority of promoters recovered appeared most active during days three and four of growth, which for agar-grown bacteria is likely to be a metabolically less-active stage. This illustrates the importance of assessing the DFI ratio in addition to the mean fluorescence data in determining the relative activity of the promoters. In order to quantify the kinetics of modulation-specific GFP

expression in more uniformly metabolically active cells, all five clones were grown in Stainer-

Figure 18A and B : Differential fluorescence induction (DFI) kinetics of agar-grown (A,B) and broth-grown (C,D) *B. pertussis.* The resultant ratio of GFP induction for growth +/- modulators is graphed below. DFI data is presented for agar and broth-grown BP338 and BP907and is extrapolated from three-day induction kinetics mean +/- SEM.

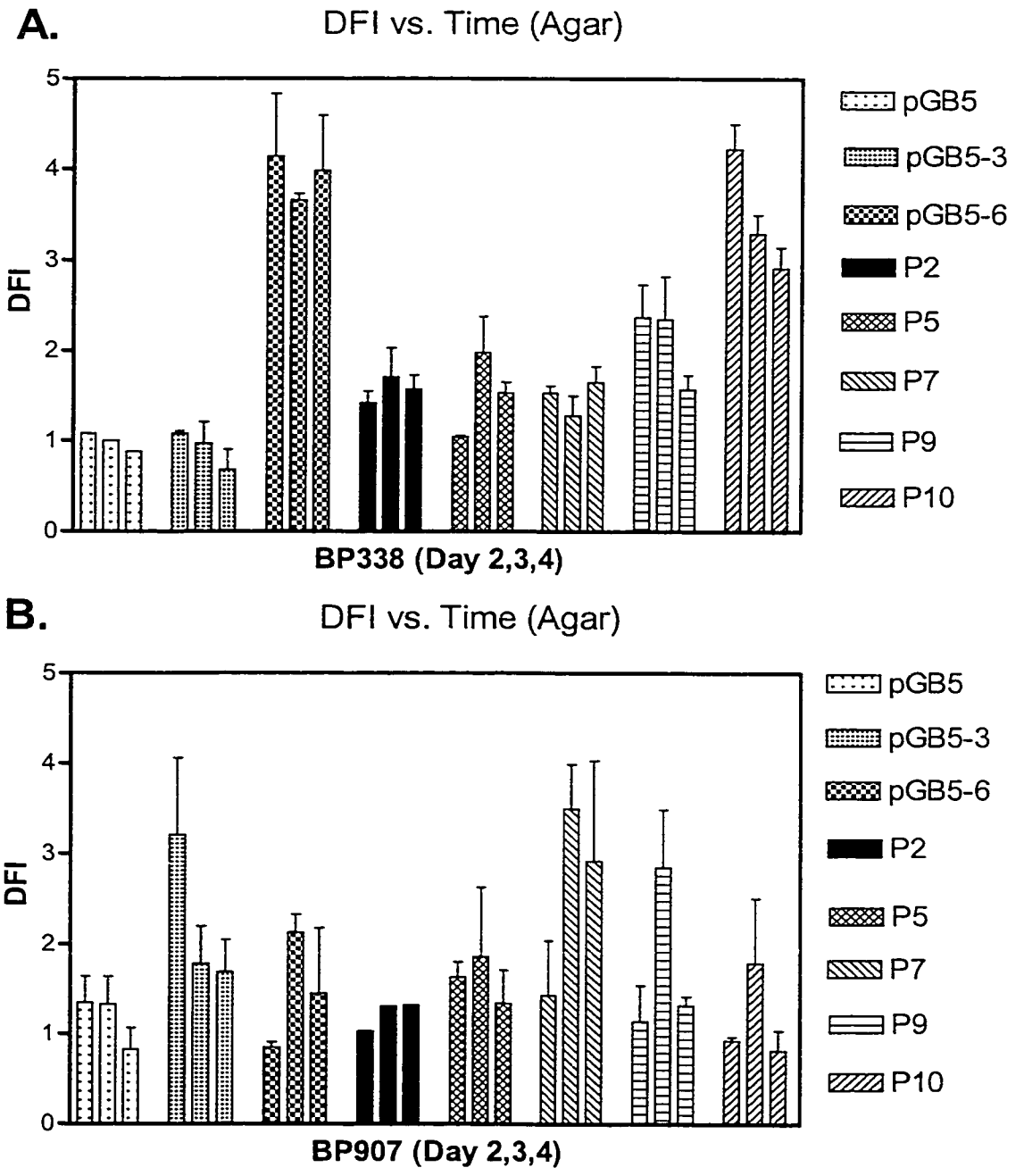
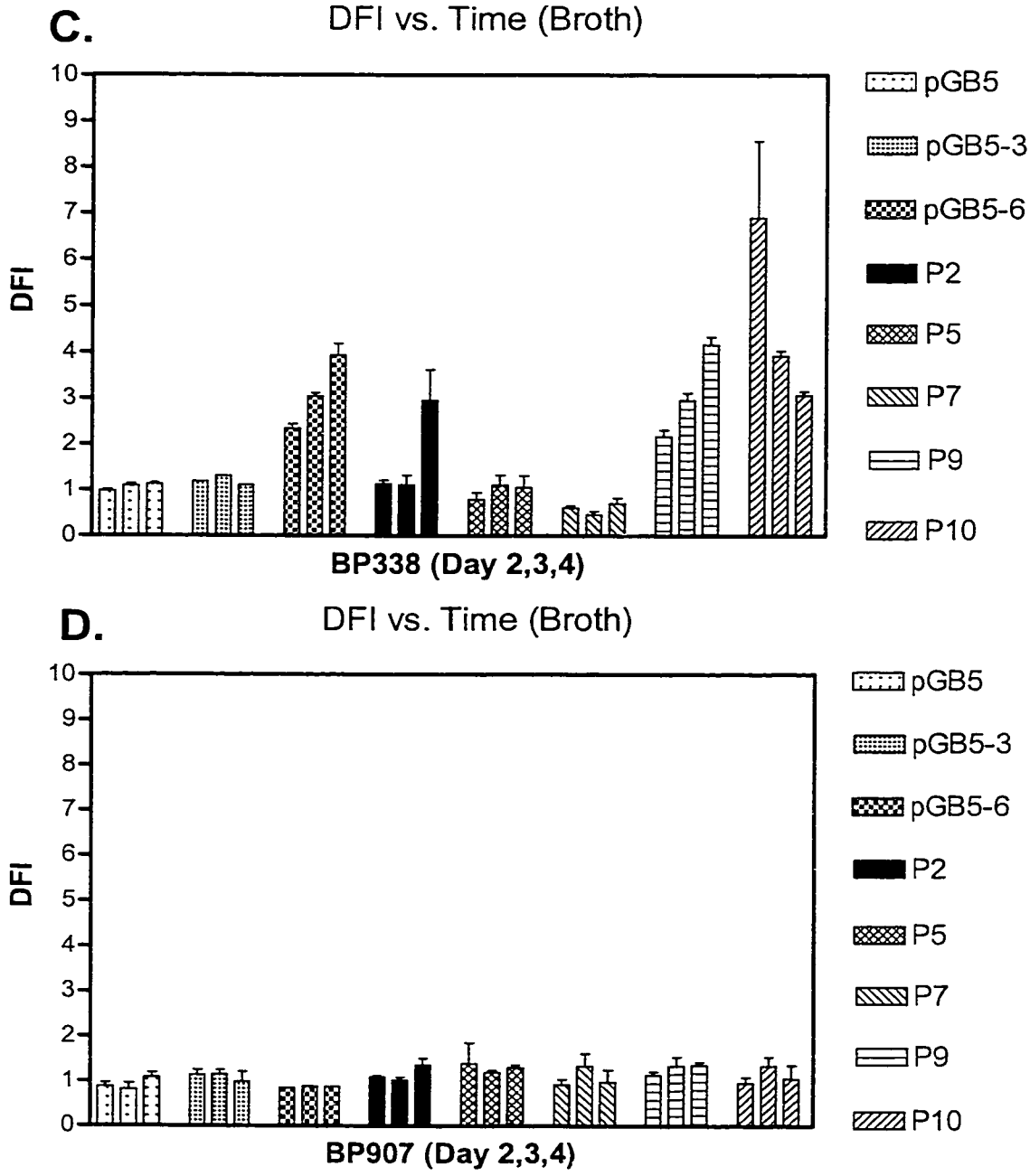


Figure 18C and D: Differential fluorescence induction (DFI) kinetics of broth-grown *B. pertussis.*



Scholte broth (SSB) and assessed for fluorescence, in triplicate, by flow cytometry over an identical three-day time course (Figures 10-17B). Interestingly, all broth-grown BP338 clones had between two-ten times higher mean fluorescence values than their agar-grown counterparts. Most notable were pGB5-P2, pGB5-P9 and pGB5-P10, which at their peaks had between 100 to 250U compared to around 20U on agar (Figures 13B, 16B, 17B). Control strains pGB5 and pGB5-3 had no significant increased expression in broth (Figures 10B, 11B). This indicates that growth in broth *per se* is not responsible for higher fluorescence readings, due to endogenous fluorescence or residual broth precipitates. In addition, a constitutively expressed promoter isolated in the course of the screen, pGB5-P1, was found to express a mean fluorescence of X=491$\pm$43 on BGA and X=613$\pm$4.3 in SSB (Only 1.2 x higher mean fluorescence in broth). This constitutive clone did not, therefore, show significant heightened expression when grown in SSB. This supports the idea that the decreased expression on agar is likely not a general phenomenon for all *B. pertussis* genes and illustrates the significance of low agar inducibility for many modulation-specific clones.

In addition to expressing higher levels of GFP in SSB with modulators, all clones had significantly different temporal reporter expression as illustrated by the DFI peaks. (Figures 10-17B and17C). Three clones (pGB5-P2, pGB5-P9, and pGB5-P10) had up to three-fold higher DFI levels in broth as compared to agar growth and showed definite temporal expression (compare Figures 18A and 18C). For two clones, pGB5-P2 and pGB5-P9 there was a marked increase in

fold induction as the time course progressed. This was mimicked in pGB5-6, which had a ladder-like progression in fold-induction from day two through four (Figure 18C). Another clone, pGB5-P10 had the opposite progression and peaked early then steadily decreased in DFI. Interestingly, this clone was the only one to demonstrate similar DFI expression patterns in agar- and broth, for the others there were marked differences (compare Figures 18A and 18C). Surprisingly, pGB5-P5 and pGB5-P7 showed increased GFP expression in broth with no modulators, despite *bvg*-repressed expression on agar (Figures 14B, 15B). This was a truly unexpected result, but may further strengthen the argument that growth media alters the expression of modulation-specific determinants. In general, growth in broth was found to be a much more sensitive and consistent means by which to quantify fluorescence levels over time. Substantial increases and decreases in DFI over the three-day time course were observed for clones pGB5-6, pGB5-P2, pGB5-P9 and pGB5-P10, whereas pGB5-P5 and pGB5-P7 displayed an unexpected *bvg*-activated induction when grown in broth (12B, 13B).

3.3 Growth curve analysis of modulated and virulent *B. pertussis.*

In previous studies with *B. pertussis,* virulence gene expression has been associated with a growth disadvantage on BGA, presumably due to self-toxicity or energy expenditure (Weiss and Hewlett 1983). Indeed, colonies growing on BGA are smaller than those grown on BGA with modulators (Lacey 1960). However, the effect of modulation on growth rate in liquid broth has not been

determined. Although the differences in GFP expression were likely not to be related to the metabolic activity of the cells, since four day old broth cultures still expressed high levels of GFP compared to agar-grown cultures, it remained possible that growth rates were affected. In order to ensure that the observed GFP expression kinetics in broth-grown bacteria did not reflect a growth rate difference between virulent *vs.* modulated *B. pertussis*, a standard growth curve assay was performed in Stainer-Scholte broth (Figure 19A). Cultures of BP338 (pGB5) were grown under the same conditions as for FACS analysis and serial dilutions were plated onto selective media over a three-day time course in broth. Both the optical density and plate counts confirmed that there was no significant growth advantage for modulated bacteria: Both non-modulated and modulated cultures grew at similar rates over the course of the experiment (Figure 19A and 19B.)

In order to ensure that GFP expression was not detrimental to bacterial growth, a growth curve was obtained using BP338 pGB5-3 and BP338 pGB5-6. BP338 pGB5-3 and BP338 pGB5-6 were chosen because they have the same promoter sequence but only pGB5-6 is a productive GFP fusion. If GFP expression were detrimental to bacterial growth then there would likely be a marked difference between pGB5-3 and pGB5-6 growth under modulating conditions. As evident in Figure 19C and 19D, no significant growth rate difference was apparent between fluorescent or non-fluorescent cultures in SSB or SSB with modulators (Figure 19C and 19D). Thus neither GFP expression, nor

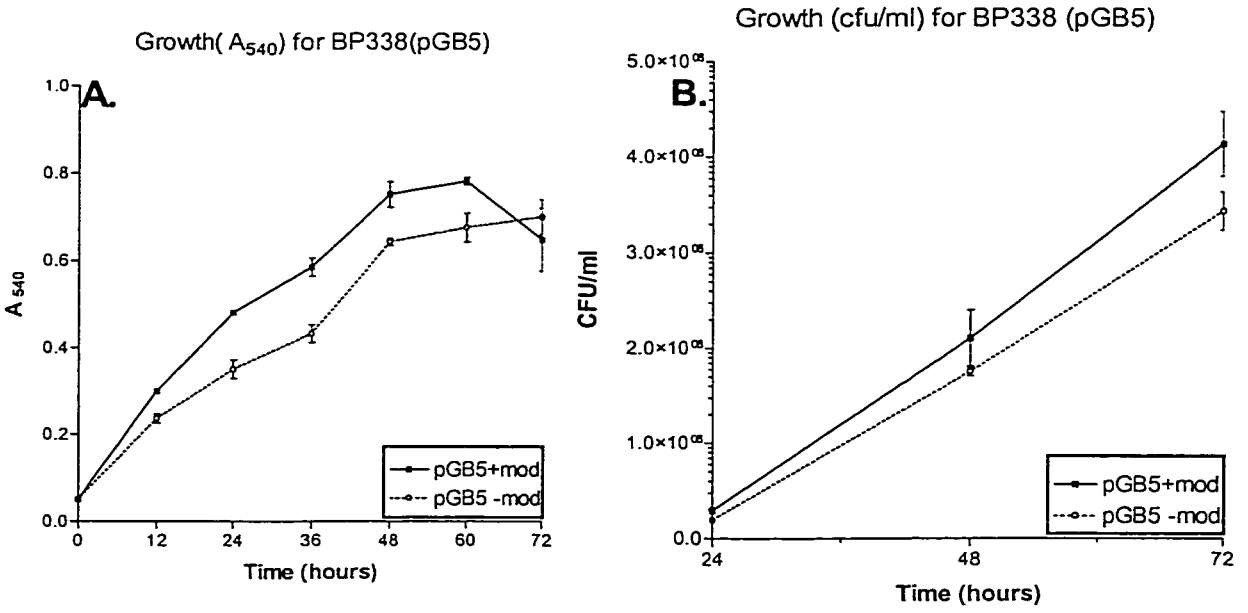


Figure 19A and B: Growth curve and cfu/ml for modulated and virulent BP338(pGB5) strains. Bacteria were grown to stationary phase in SSB with and without modulators (+ mod, -mod respectively) and were sampled in triplicate at multiple time points during a 72 hour time period for analysis. Serial dilutions from the SSB were plated on BGA to determine cfu/ml and spectrophotometer readings were taken at A_{540} to generate a standard growth curve. Initial inoculum was 0.05 OD_{540}

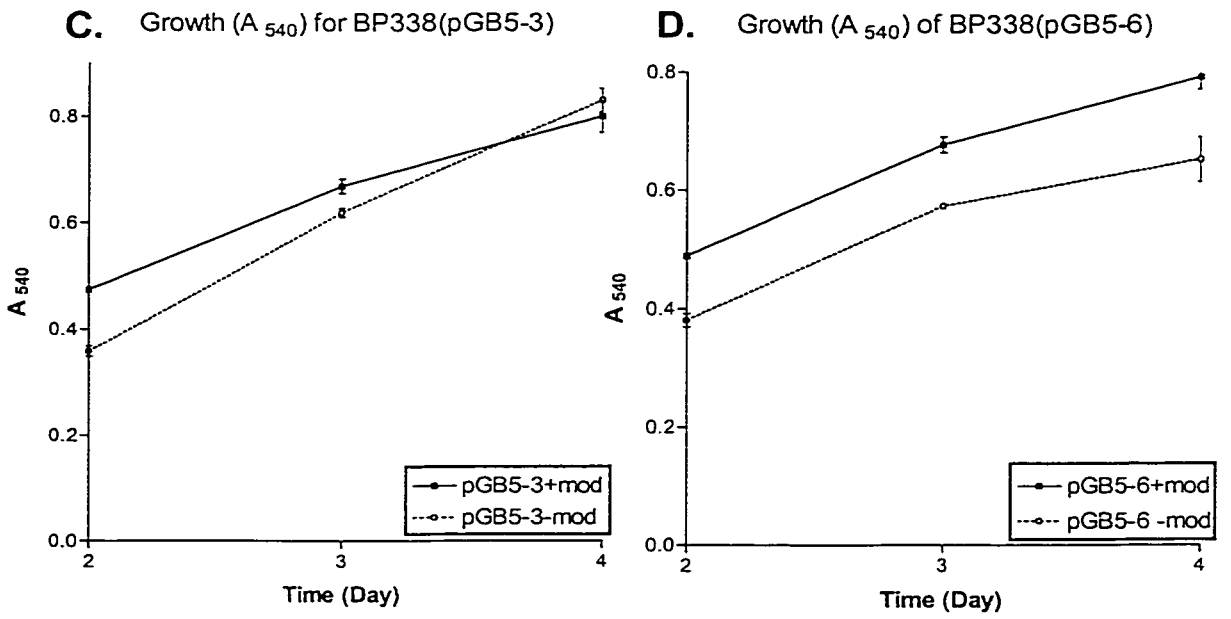


Figure 19 C and D: Growth curve of BP338 (pGB5-3) and BP338 (pGB5-6) in SSB with and without modulators (+mod and -mod respectively). Bacteria were sampled in triplicate at multiple time points and read by the spectrophoptometer at $A=_{540}$ during a 72 hour time period to generate the growth curve. Initial inoculum was 0.05 OD $_{540}$

modulation had any significant effect on growth rate or viability of the constructed strains.

3.4 Role of BvgAS in regulation of modulation-specific promoters.

Although all five clones were selected by virtue of their ability to preferentially express GFP under modulating conditions, it remained unknown whether the main virulence regulator, BvgAS was involved in GFP regulation. Modulation-specific promoters under the control of BvgAS would be expected to show constitutive GFP expression in a *bvg*A$^-$ background. In order to test for BvgA dependence, the GFP promoter fusions (pGB5 plasmids) were transformed into the *bvg*A knockout strain, BP907, and tested for constitutive GFP expression by FACS analysis (Figures 10-17C and D). Once again, the results for agar- and broth-grown cultures differ. Due to this discrepancy only BP338 and BP907 strains grown under like conditions will be directly compared and discussed.

All BP907 strains expressed elevated levels of GFP when grown on agar compared to BP338 strains (compare Figures 10-17A and C). This high level of expression could be explained by the accumulation of GFP under constitutive expression conditions. It was unexpected, however, that there was increased GFP expression on BGA with modulators for all clones, including the control strains containing pGB5-6 (*vrg6::gfp*). This was peculiar since the mutation to the BvgA protein in the BP907 background abolishes the BvgAS phosphorelay cascade, which is required to repress *bvg*-repressed genes and activate *bvg*-activated ones. If the bacteria are still responding to modulators in BGA, then it

is likely that modulating signals may be acting on additional regulatory cascades that are triggered when bacteria are specifically grown on agar. This was most evident for strains BP907 pGB5-7 and pGB5-P9, which exhibited four and three-fold modulation-specific GFP expression, in spite of the *bvg*A mutation in BP907 (Figures15C and 16C). Although these BvgAS non-responsive clones did in fact still respond to modulators in agar, the degree of induction was reduced in some clones by as much as 60% in the BP907 background, which at least indicates that the mutation has some effect on *bvg*-repressed gene expression. For example, BP338 pGB5-6 and pGB5-P10 dropped from approximately 5-fold induction (BP338) down to 2-fold in the 907 strain (compare Figures 17A and 17B).

Compared to the peculiar modulation responsiveness of certain clones when grown on agar, the broth-grown cultures were more predictable (Figures 10-17D). The clones, which showed modulation-specific induction in SSB in the BP338 background, namely pGB5-P2, pGB5-P9 and pGB5-P10 showed the expected constitutive expression of GFP in the BP907 background. This indicates that theses clones are under BvgAS control (compare Figure 17 C-D). This was consistent with the results for the control strain, BP907 pGB5-6, which mimicked the constitutive expression in SSB, despite modulation specific induction when grown on agar (compare Figure 12C and 12D). The strain BP907 (pGB5-P7) was perhaps the most intriguing strain discovered in the screen, as it expressed 3.5 fold more GFP when grown on agar with modulators, but showed no differential expression in broth (compare Figure 14C and 14D). Although

BP338 pGB5 P5 was only able to induce modulation-specific expression of GFP on agar, BP907 pGB5 P5 preferentially expressed GFP in both agar- and broth-culture with modulators (compare Figure 14 A and 14D).

If the experimental strains are compared to results from the control strain with plasmid pGB5-6, then pGB5-P2, pGB5-P9 and pGB5-P10 all behave like BvgAS-regulated, modulation-specific promoters. The promoter in pGB5-P5 could not properly be assessed since it only showed *bvg*-repressed expression on agar and agar-grown BP907 strains all showed induction under modulating conditions. The promoter pGB5-P7 was intriguing in that it showed *bvg*-activated induction in broth, which was abolished in the BP907 strain. This may indicate control by a novel repressor that is expressed under modulating conditions and regulated by BvgAS.

3.5 Effect of increased osmolarity on DFI

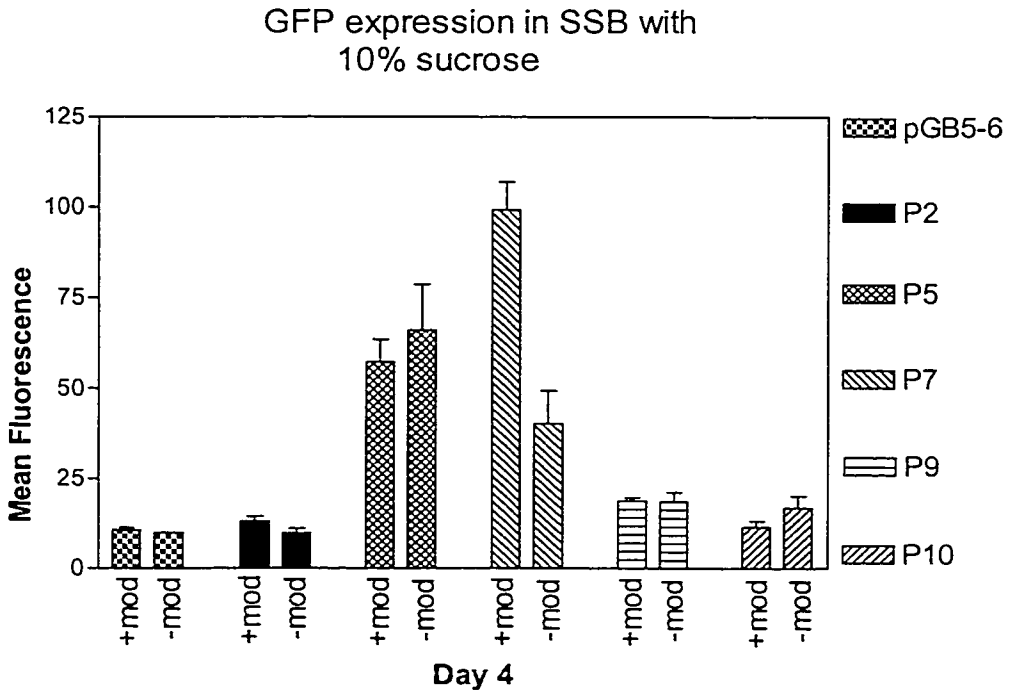
In order to determine whether osmolarity differences between broth and agar media may be responsible for the lowered GFP induction levels for agar-grown bacteria in general, the clones were grown statically in high osmolarity broth (supplemented with 10% sucrose) with and without modulators. Sucrose was chosen since the organism cannot metabolize it and bacterial growth did not seem to be adversely effected. This was in marked contrast to the use of SSB +5% NaCl, which did not support bacterial growth. Afterfour days the clones were subjected to FACS analysis and the results are presented in Figure 20. For three putative BvgAS regulated factors (pGB5-P2, pGB5-P9 and pGB5-P10) grown in high osmolarity medium, fluorescence was substantially

diminished and overall levels were comparable to those seen in pGB5-6 (Figure 12A). This strongly suggests that the osmolarity and perhaps static growth conditions adversely effect expression of BvgAS-regulated promoter fusions. The other, non BvgAS-regulated clones, pGB5-P5 and pGB5-P7 were still able to express high levels of GFP in static, high osmolarity growth conditions, indicating that this was not a general phenomenon but rather specific to the BvgAS regulon. The strain BP338 pGB5-P5 showed similar GFP expression in high osmolarity broth as compared with regular SSB, indicating that the observed modulation-specific GFP induction in agar is likely not to be osmolarity-dependent for this strain (compare Figure 14B and 20). For BP338 pGB5-P7 modulation–specific expression in SSB with 10% sucrose was higher than for any other clone tested. Recall that this clone only expressed modulation-specific induction when grown on agar and in broth it was induced under regular growth conditions. Growth in SSB+10% sucrose and modulators mimicked the modulation-specific induction evident in BGA with modulators indicating a connection between modulation-specific regulation, osmolarity and inducibility for this strain.

3.6 Sequence and analysis of modulation-specific promoters.

All clones were partially sequenced and searched against the unfinished *B. pertussis* genome using the basic Blast algorithm. All partial sequences were mapped to contigs which were subsequently searched against all known sequence databases using the Blast-X algorithm in order to identify possible

Figure 20: GFP induction in SSB with 10% sucrose. GFP induction in high osmolarity broth was tested to determine whether osmolarity differences could account for diminished fluorescence of *bvg*-repressed genes in agar *vs.* broth medium. Bacteria were grown statically for four-days in 6-well microtitre trays in SSB +10% sucrose in the presence and absence of modulators (+mod and -mod respectively). SSB+ 10% sucrose-grown bacteria were FACS-scanned in triplicate. Data are represented as the mean of 5000 bacteria +/-SEM.



open reading frames (ORFs) and possible promoter sequences. Figures 21-25A show the partial captured upstream sequences relative to GFP for all pGB5 clones and indicate whether they contain putative ORFs, promoters or both.

Clones pGB5-P2, pGB5-P5, and pGB5-P10 captured putative promoter sequences that had downstream ORFs with homology to known proteins. Clone pGB5-P2 captured a putative promoter with downstream sequence homology (P value of 2e-52, as explained at the Blast site) to a D-isomer specific 2-hydroxyacid dehydrogenase from several bacterial and eukaryotic species starting at amino acids 20 onward. The protein homology is diagrammed in Figure 21B. A Blast-X search with captured sequence upstream of GFP (diagrammed in Figure 21A) failed to identify an ORF, making it a candidate promoter. Although the first 20 AA of the protein were not identified in the Blast search, it remains possible that they are present at least in part in the captured upstream "promoter" region. There are several in-frame start sites that could act as initiator codons in this region and a strong consensus BvgR binding site that could account for modulation specific expression.

Clone pGB5-P5 captured a putative promoter and partial-58 amino acid ORF (Figure 22A). Again the ORF was incomplete and lacked the first 7 amino acids, which could be further upstream and mismatched. The putative upstream promoter sequence had no downstream homology to known proteins and there were several in-frame initiator codons present. The downstream sequence had high homology to a 5' inosine-monophosphate dehydrogenase (IMP dehydogenase) from several bacterial and eukaryotic species, most notably

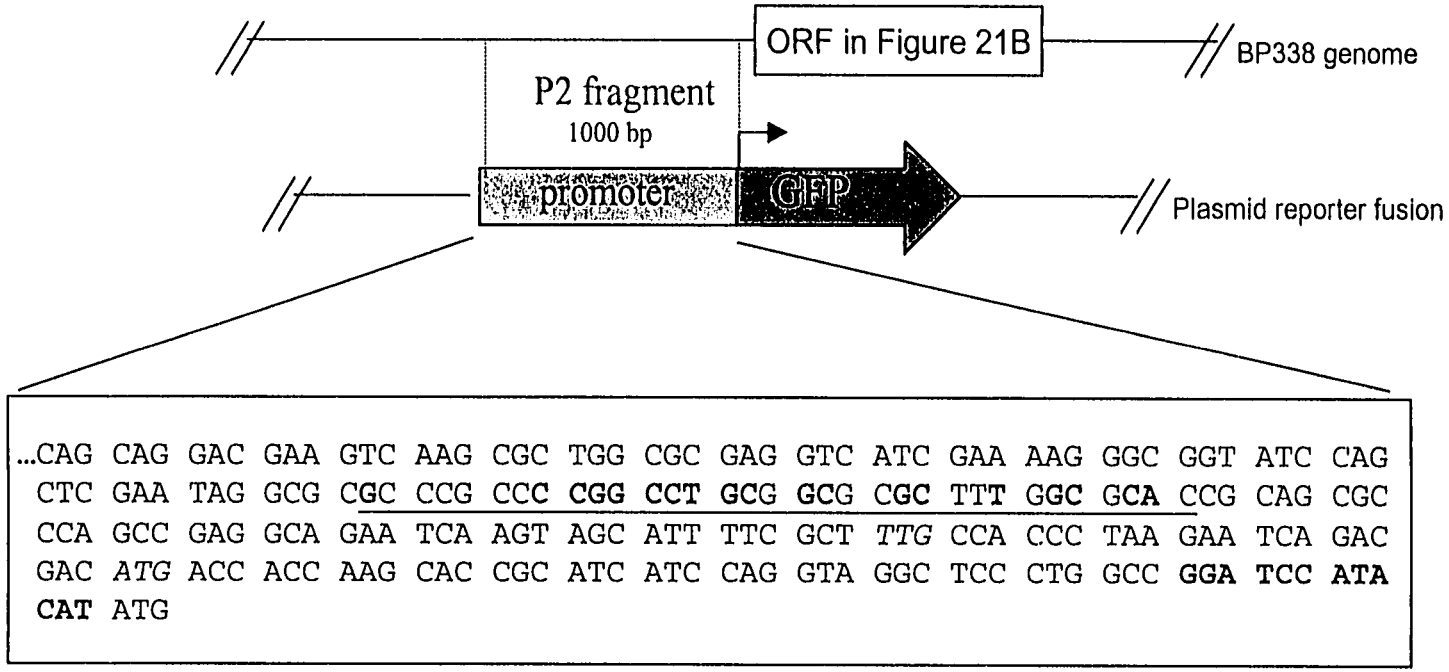


Figure 21A. DNA fragment upstream of GFP in the clone pGB5-P2. A partial sequence of clone pGB5-P2 was obtained using a backwards facing primer from GFP. The sequence shown above was searched against the partial *B pertussis* unfinished genome sequence and found to map to contig 88 between sections 8903-8791. The entire contig contains the partial putative coding region (from amino acids 20-262 of 313) and promoter sequence of a D-isomer specific 2-hydroxyacid dehydrogenase. The clone captured the putative promoter sequence but no known ORF. Since the protein match was only from amino acid 20 onward, it is possible that some additional amino acids are encoded in the putative promoter sequence. The ribosome binding site from the vector is in **bold-face,not underlined** and the possible start is in *italics*. The putative BvgR binding site is **underlined** with consensus matches in **bold-face**

```
P2 protein: 24  LADEYDVLELWQQADRAAALARHGKGVTALVTSANFGXXXXXXXXXXXXXXICSWGVGYE
                L   +++L  W   +++  L  H   + A+V +A+ G              + S+ VG +
HDH-A       20  LEKRFNLLRFWTSPEKSVLLETHRNSIRAVVGNASAGADAQLISDLPNLEIVSSFSVGLD

P2 protein: 204 TIDVDAARKRGVLVSNTPDVLTDCVADLAWGLLISGARRMAQGDRFVRAGQWGQVHGSIP
                 ID+   +++G+ V+NTPDVLT+ VADLA GL+++  RR+ + DR+VR+G+W Q  G
HDH-A       80  KIDLGKCKEKGIRVTNTPDVLTEDVADLAIGLILALLRRLCECDRYVRSGKWKQ--GEFQ

P2 protein: 384 LGTRVSGKNLGIIGLGRIGEAIARRGDGFDMQVRYHNRRRRDDVSYGYESSLADLARWAD
                L T+ SGK++GIIGLGRIG AIA+R + F   + Y++R  + DV+Y Y  ++ DLA+  D
HDH-A       138 LTTKFSGKSVGIIGLGRIGTAIAKRAEAFSCPINYYSRTIKPDVAYKYYPTVVDLAQNSD

P2 protein: 564 FLVVATVGGPSTRHLVNQEVLEALGPKGLIINIARGPVIDEXXXXXXXXXXXXXXXXXDV
                 LVVA      TRH+V+++V++ALG KG++INI RGP +DE                DV
subject:    198 ILVVACPLTEQTRHIVDRQVMDALGAKGVLINIGRGPHVDEQELIKALTEGRLGGAALDV

P2 protein: 744 FEHEP
                FE EP
subject:    258 FEQEP
```

Figure 21B: Alignment of the P2 protein and the putative D-isomer specific 2-hydroxyacid dehydrogenase from *Arabidopsis* sp.

The DNA sequence downstream of the GFP cloning junction was searched using the BLAST-X algorithm and yielded a partial protein match with p value= 2e-52 for a putative D-isomer specific 2-hydroxyacid dehydrogenase from several species, however the highest match from *Arabidopsis* is shown above. The proteins shared 97/245 exact amino acid matches (39%) and 148/245 (59%) like amino acids. The full protein is 313 amino acids.

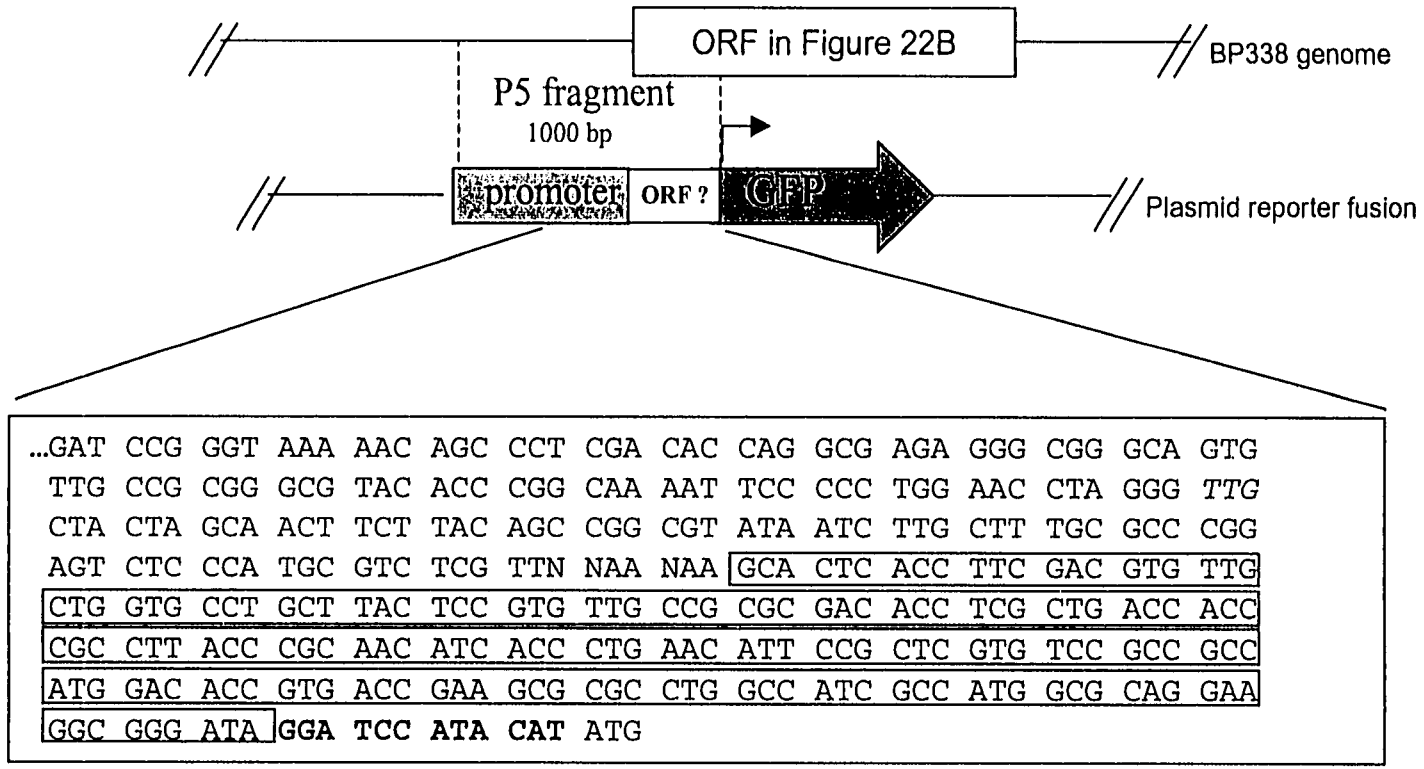


Figure 22A. DNA fragment upstream of GFP in the clone pGB5-P5. A partial sequence of clone pGB5-P5 was obtained using a backwards facing primer from GFP. The sequence shown above was searched against the partial *B pertussis* unfinished genome sequence and found to map to contig 247 between sections 30710-30976. The entire contig contains the partial putative coding region (from amino acids 7-402 of 487) and promoter sequence of a 5'inosine-monophosphate dehydrogenase. The clone pGB5-P5 captured 58 amino acids of the putative IMP dehyraogenase ORF and approximately 800bp of upstream sequence. The putative ORF is boxed and the ribosome binding site from the expression vector is in **bold-face** and the possible start in in *italics*. It is a strong possibility that a further upstream start codon and ribosome binding site are utilized in this clone.

```
P5 protein: 1182 ALTFDDVLLVPAYSEVLPRDTSXXXXXXXXXXXXIPLVSAAMDTVTEARLAIAMAQEGGI
                 A TFDDVLLVPA+S VLPRD              +PL+SAAMDTVTEARLAI+MAQEGGI
IMDH          7  AYTFDDVLLVPAHSTVLPRDVKLQTKLTREITLNLPLLSAAMDTVTEARLAISMAQEGGI

P5 protein: 1002 GIIHKNLSADDQAKEVARVKRHEFGIVIDPVTVTPDMKVRDAIAL--QRQKGISGLPVVE
                 GIIHKN+  + QA+ +++VKRHE G+V DPVTV P   +R+ + +  QR+  +SGLPVVE
IMDH          67 GIIHKNMPPEMQARAISKVKRHESGVVKDPVTVAPTTLIREVLEMRAQRKRKMSGLPVVE

P5 protein: 828  GRKLVGIVTNRDLRFEDRLDQPLRNIMTPQDRLVTMKEGATLDEAQALMHKHRLERVLIV
                   K+VGIVTNRDLRFE+R+D P+  IMTP++RLVT+ EG ++DEA+ LMH H++ERVL++
IMDH          127 NGKVVGIVTNRDLRFENRVDLPVSAIMTPRERLVTVPEGTSIDEARELMHTHKVERVLVL

P5 protein: 648  NDAFELRGLATVKDIVKNTEHPYACKDSQGQLRXXXXXXXXXGTEXRVEKLVAAGVDVII
                 N+  EL+GL TVKDI+K TE P A KDS+G+LR          TE RV+ LV AGVDVI+
IMDH          187 NEKDELKGLITVKDILKTTEFPNANKDSEGRLRVGAAVGTGGDTEERVKALVEAGVDVIV

P5 protein: 468  VDTAHGHSAGVLXRVRWVKQNYPKVEVIXXXXXXXXXXXXXXXXXXXGVKVGIGOGSICT
                 VDTAHGHS GV+ RVRWVK+ YP ++VI                    VKVGIGPGSICT
IMDH :        247 VDTAHGHSQGVIDRVRWVKETYPHIQVIGGNIATAKAALDLVAAGADAVKVGIGPGSICT

P5 protein: 288  TRIVAGVGVPQISAISDVAKALEGTGVPLIADGGIRYSGDVXXXXXXXXXXXXXXXXXXG
                 TRIVAGVGVPQ++AI +VA+AL+GTGVPLIADGGIR+SGD+                 G
IMDH          307 TRIVAGVGVPQLTAIHNVAEALKGTGVPLIADGGIRFSGDIAKALAAGAYSVMLGGMFAG

P5 protein: 108  TEEAPGEVVLFQGRSYKSYRGMGSLGAMADGSADRY
                 TEEAPGE+ L+QGRSYKSYRGMGSLGAM+ GSADRY
IMDH          367 TEEAPGEIELYQGRSYKSYRGMGSLGAMSQGSADRY
```

Figure 22B: Alignment of the P5 protein and the putative inosine-5'-monophosphate dehydrogenase from *Neissieria meningitidis*. The DNA sequence downstream of the GFP cloning junction was searched using the BLAST-X algorithm and yielded a partial protein match with p value=e -130 from several bacterial species, however the highest match from *Neisseria* is shown above. The proteins shared 238/394 exact amino acid matches (60%) and 290/394 (73%) like amino acids. The full protein is 487 amino acids and is contained on an unfinished cosmid and therefore was not searched in entirety. The 58 amino acids captured in pGB5-P5 are in **boldface**

similar to that from *Neisseria meningitidis* (P value= e-130; Figure 22B). There was an apparent lack of a BvgR consensus-binding site, which is consistent with this agar responsive modulation-specific promoter being independent of BvgAS control.

Clone pGB5-P10 captured the known promoter and partial ORF along with the BvgR binding site of the previously identified *B. pertussis bvg*-repressed gene, *vrg6* (Figure 23A and 23B). Although this identified the same locus as constructed for the control strain pGB5-6, the captured promoter was more responsive to growth in SSB with modulators. Since the captured promoter was larger than that in the constructed strain it is likely that upstream elements are responsible for enhanced expression and activation in this promoter construct.

The remaining two clones were less straightforward. Clone pGB5-P7 captured a partial ORF with homology to a branched chain amino acid transporter ATP binding protein at amino acids 206-240 but no known promoter sequence (Figure 24A and 24B). Contrary to this, there is a good consensus BvgR binding site upstream of the cloning junction and several in-frame start codons. It is unknown whether this sequence is a *bona fide* promoter in *B. pertussis*, but it is clear that it is capable of driving GFP expression in a modulation-specific manner on plates and vice versa in broth. It is unknown if the ATP binding protein is expressed in the organism.

Clone pGB5-P9 also captured a putative ORF with limited homology to a hypothetical cardiolipin synthase from *Bacillus subtilis* but only at 25% over 75 amino acids when searched with the entire contig (see Figure 25A and 25B).

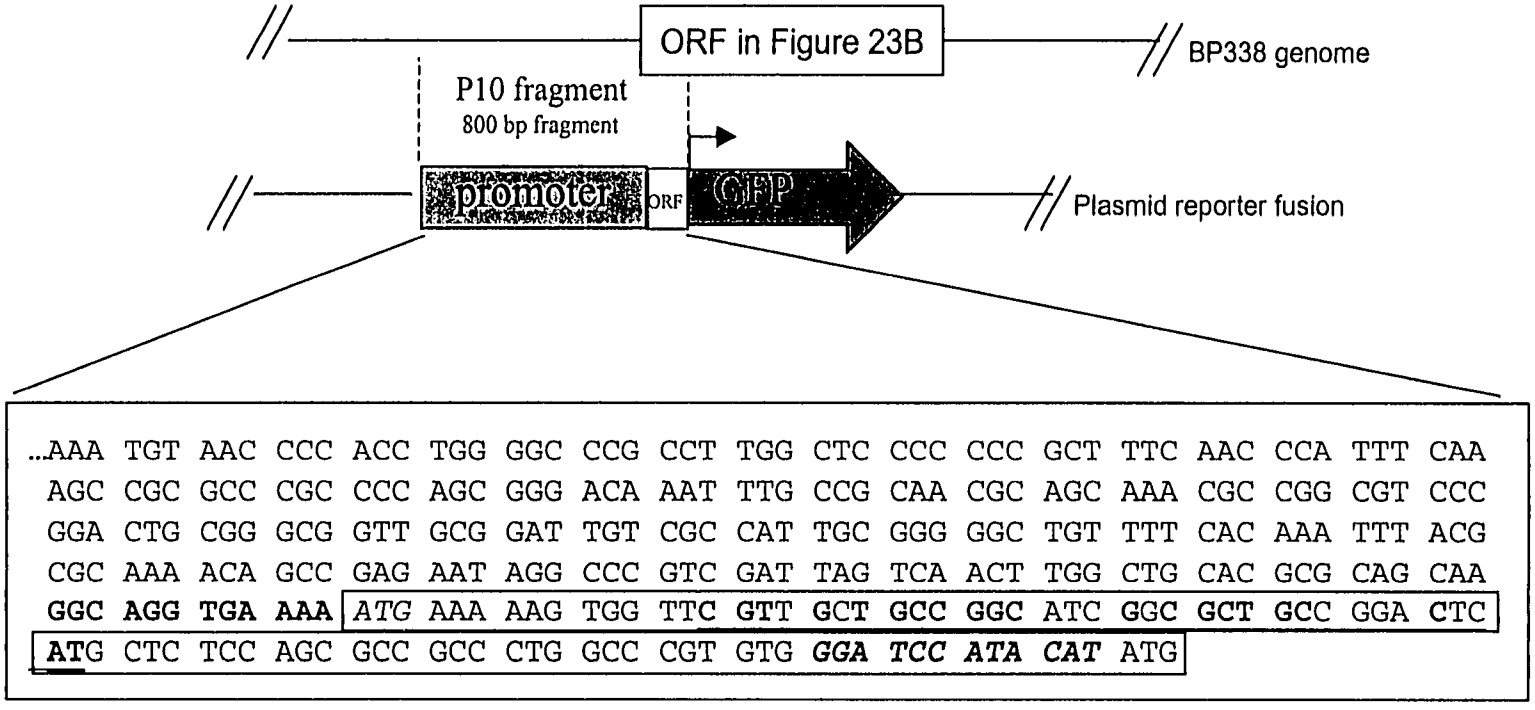


Figure 23A. DNA fragment upstream of GFP in the clone pGB5-P10. The complete sequence of clone pGB5-P10 was obtained using a backwards facing primer from GFP. The sequence shown above was searched against the partial *B pertussis* unfinished genome sequence and found to map to contig 302 between 66880-67226. The contig contains the promoter sequence and partial coding region of the known Bvg-regulated gene *vrg-6*. The clone captured the promoter and partial ORF that contains the BvgR binding site. The start codon is in *italics*. The known BvgR binding site is underlined, the BvgR binding site consensus is in **bold-face** and the partial ORF is boxed. The known ribosome binding site precedes the start codon, ATG, and is in Bold face and the vector ribosome binding site is in bold and italics, not underlined.

```
P10 protein: 996 MKKWFVAAGIGAAGLMLSSAALARVDIGVSIGIPGVVYPAPVYVAPAPVYAPPPVVHYPA
                 MKKWFVAAGIGAAGLMLSSAALARVDIGVSIGIPGVVYPAPVYVAPAPVYAPPPVVHYPA
Vrg6   :   1     MKKWFVAAGIGAAGLMLSSAALARVDIGVSIGIPGVVYPAPVYVAPAPVYAPPPVVHYPA

P10 protein: 816 PVYVRPQVVYPAPVYYGGPRYYKGHRHYDRGHRGHRGHGRGHWRD
                 PVYVRPQVVYPAPVYYGGPRYYKGHRHYDRGHRGHRGHGRGHWRD
Vrg6        61   PVYVRPQVVYPAPVYYGGPRYYKGHRHYDRGHRGHRGHGRGHWRD
```

Figure 23B: Alignment of the P10 protein Vrg6 from *Bordetella pertussis* . The DNA sequence around the GFP cloning junction was searched using the BLAST-X algorithm and yielded a total protein match with p value=4e-60 to the Vrg6 protein from *Bordetella pertussis.* The proteins shared 105/105 exact amino acid matches (100%) of a possible 105 amino acids . The partial ORF captured in clone pGB5-P10 is in **boldface.**

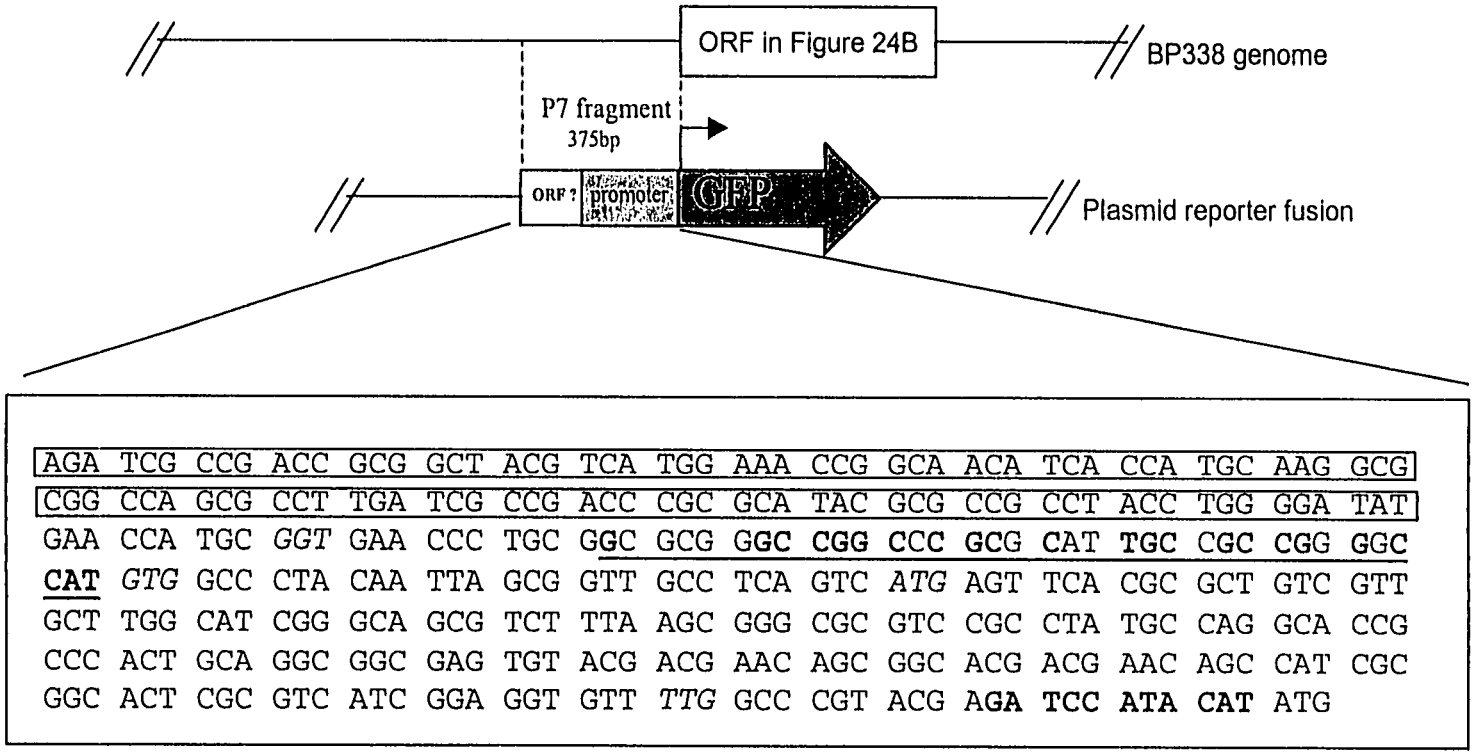


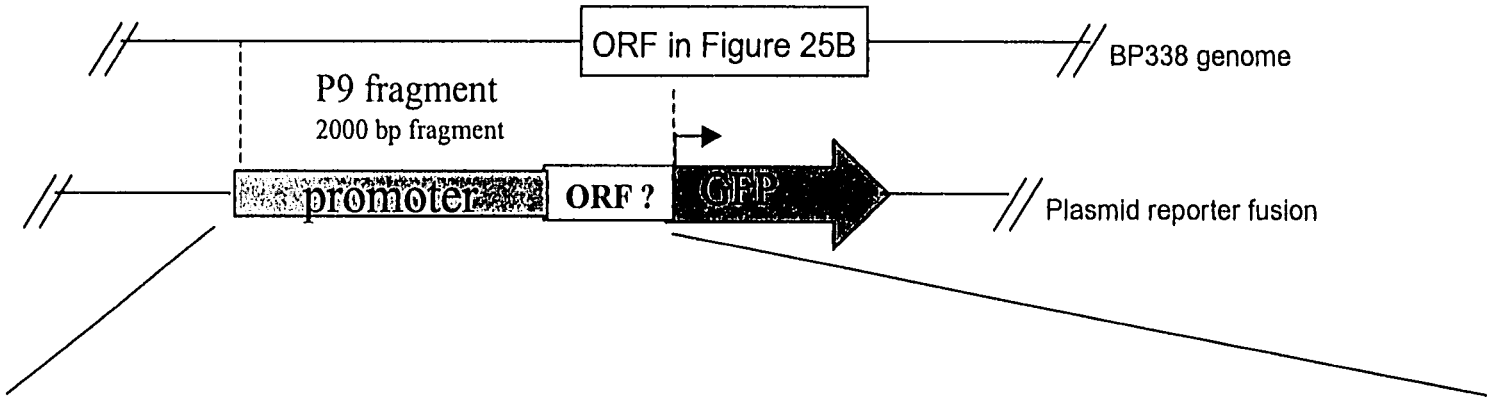
Figure 24A. DNA fragment upstream of GFP in the clone pGB5-P7. The complete sequence of clone pGB5-P7 was obtained using a backwards facing primer from GFP. The sequence shown above was searched against the partial *B pertussis* unfinished genome sequence and found to map to contig 293 between 18953-18655. The contig contains the entire putative coding region (amino acids 1-232/233) and presumably a partial promoter sequence of a branched-chain amino acid ABC transport ATP-binding protein (braG) from *Pseudomonas* as well as a hypothetical transcriptional regulator from *Bacillus*, yuxN. The clone is complex in that it captured the end part of the predicted ABC transporter ORF (amino acid 206-232/233) and the promoter of the hypothetical transcriptional regulator. The putative ORF is boxed, the ribosome binding site is in **bold-face,** not unlined and possible start codons are in *italics.* The putative BvgR binding site is **underlined** and the BvgR binding site consensus is in **bold-face.**

```
P7 protein: 29    IIERAAAMFARQGYSETSIGDIARACECSKSRLYHYFDSKEAVLRDMLTTHVDSLLERCR
                  IIE A  +FA++GY+ TS  +IA+ C+ SK   Y YF SKEA+L  ML   +D    R
YuxN       8      IIETALKLFAQKGYNSTSVQEIAKECKISKGAFYIYFPSKEALLLSMLNYYYDKTFTRIL

P7 protein: 209   QVLYGSNEPKTRFLQIVKLFLEIYATSRDRHVVMLTCLDALPEDQRKALIAKQRELIAYV
                   +    + P+T + + + +  E     +D  + M     +LP  +     AK+      +
YuxN  :    68     NIKTKGDSPRTAYRKQLTVLYENILEHKD-FISMQLKEGSLPYTEEVEQCAKK------I

P7 protein: 389   RDALLQLRPDMAAN
                  R + LQ   D   N
YuxN     121      RQSSLQFHIDSLLN
```

Figure 24B: Alignment of the P7 protein and the hypothetical transcriptional regulator from *Bacillus subtilis*, YuxN. The DNA sequence downstream of the GFP cloning junction was searched using the BLAST-X algorithm and yielded a partial protein match with p value=5e -09 from several bacterial species, however the highest match from *Bacillus* is shown above. The proteins shared 41/134 exact amino acid matches (30%) and 65/134 (47%) like amino acids. The full protein is 291 amino acids and is contained on an unfinished cosmid and therefore was not searched in entirety.



...ATT GAC ACG CCC GCG **CCG GCG** TGC **GCC** GGC **CTG** CTC CGG **GCG** GCG GAA ATA CTG CTT
TAC CGC TGG TTG CCA AAC CAG AGG CGT GCG GCG CGG GCC CCG GTC AGA ATA CGG GCG
TGT CAT AAG GCC AAG TGG GAG ATT CGC TAT GTC GTC GAT CGT GGT GGC ACG GTT CGA
TAG CGT CCC TTC GGC GAG AAG CGC TGC GCA CGC GTT GGT CGG CGA CGG CTT TCG CGA
AGA GGC AAT CAG TAT CCT GTA CGG CGA CCC **GGA TCC ATA CAT** ATG

Figure 25A. DNA fragment upstream of GFP in the clone pGB5-P9. The complete sequence of clone pGB5-P9 was obtained using a backwards facing primer from GFP. The sequence shown above was searched against the partial *B pertussis* unfinished genome sequence and found to map to contig 231 between 25332-25749. The contig contains very limited homology to the partial putative coding region of a cardiolipin synthase. The clone captured part of the predicted ORF (amino acids 119-193/ 482). It is unknown whether the sequence functions as a *bona fide* promoter in *B. pertussis*, however the size of the fragment is large and upstream sequences may be responsible for GFP expression. The vector ribosome binding site is in **bold-face**, not underlined. The putative BvgR binding site is **underlined** and the BvgR binding site consensus is in **bold-face.**

```
P9 protein 996   NQVRLLADGPATYRSMLDAIAQARRYIHMETYIFEDDEAGQRFADALMAARQRGVEVAVM
                 N V ++ DG    ++ +L   I++A+   +H++ YI++ DE G++   DAL+     + GV+V V+
cls:       1     NSVDVITDGRDKFQRLLSDISKAKDHIHLQYYIYKGDELGKKLRDALIQKAKEGVQVRVL

P9 protein: 816  VDAVGTLNTPRQWFE
                  D +G+     +++F+
cls:       61    YDELGSRTLRKKFFK
```

Figure 25B: Alignment of the P9 protein and the hypothetical cardiolipin synthase from *Bacillus subtilis*. The DNA sequence upstream of the GFP cloning junction was searched using the BLAST-X algorithm and yielded a partial protein match with p value=6e-07 from several bacterial species, however the highest match from *Bacillus* is shown above. The proteins shared 24/75 exact amino acid matches (32%) and 49/75 (65%) like amino acids. The full protein is 482 amino acids, however, only limited homology was apparent despite the large size of the cosmid. All 72 amino acids captured in pGB5-P9 are shown above.

Although the cardiolipin synthase protein is 482 amino acids long, the contig only showed homology to the captured region between amino acids 119-193. It is doubtful that the P9 fragment encodes this protein, but it is evident that some limited homology exists. Since the P9 fragment is a 2Kb region, there is a high probability that upstream promoter sequences are driving GFP expression but the contig failed to produce any other matches. It is clear that this region can function as a promoter and there exists a strong BvgR binding site consensus and several start codons, however it is unclear if it is a true promoter in *B. pertussis*. The identity of the P9 fragment is therefore still unknown.

4. Discussion

Bacterial pathogens produce a large variety of virulence determinants in order to carefully orchestrate an infection and spread from host to host. The ability to coordinately regulate gene expression is critical to accomplish this task. In the fastidious upper respiratory tract pathogen *B. pertussis,* coordinate virulence gene expression is dependent on the two-component regulator BvgAS. This regulator is responsible for the activation of virulence determinants and the simultaneous repression of *bvg*-repressed genes. This is achieved by the binding of BvgR to a 32 base-pair consensus in four of the five *bvg*-repressed identified previously. Although the roles of *bvg*-activated virulence determinants are well established, the contribution of the *bvg*-repressed genes in the life of *B. pertussis* is unknown. The GFP-based promoter trapping expression library was created with the goal of identifying novel *bvg*-repressed candidates that could contribute to our understanding of this elusive set of genes. This DFI strategy, initially used to isolate acid-inducible promoters in *Salmonella typhimurium* (Valdivia *et al.* 1996), was successfully applied to isolate a bank of intriguing modulation-specific promoters in *B. pertussis.* Overall, of the five clones chosen for analysis based on modulation-specific expression in agar, three were classically *bvg*-regulated (pGB5-P2, pGB5-P9 and pGB5-P10) while the remaining two were modulation specific by another mechanism.

As an alternative to methods that rely on chromosomal insertion, a "promoter trapping" approach was used. This screen offered several advantages

over conventional strategies like transposon mutagenesis which have been utilized with success in identifying both *bvg*-activated and *bvg*-repressed genes in *B. pertussis* (Weiss *et al.*, 1983);(Stenson and Peppler 1995)(Finn *et al.*, 1991). While transposon insertions sites are sensitive to sequence context and may not be totally random in *B. pertussis*, (Weiss *et al.*, 1989) partial digestion with the four base-pair recognition enzyme, *Sau*3AI, generates a largely unbiased library of fragments. This was important for identifying novel genes that previous Tn-based screens may have missed, and indeed two novel *bona fide* *bvg*-regulated clones, pGB5-P2 and pGB5-P9 were identified. There are concerns, however, about the use of a multi-copy plasmid over a single copy transposons for gene regulation. First, the ratio of *trans*-acting factors to DNA is lower. Thus it may not adequately mimic true levels of gene expression, since regulators may be limiting. For example, there may be insufficient BvgR to adequately repress all extra-chromosomal copies. Second, plasmids exist in a supercoiled state *in vivo,* imposing a possible topological difference from the normal chromosomal context. However, the use of a plasmid-based system eliminates the potential for polar transposition effects on gene expression. In addition, single copy GFP transgene fusions appear to have sub-threshold fluorescence expression in Bordetellae (this thesis, data not shown; Dr. P.A. Cotter, personal communications).

The use of the low copy number GFP expression plasmid, pGB5, gave induction rates similar to single copy chromosome fusions for the known *bvg*-repressed gene *vrg6* (4 fold), thus demonstrating that the low copy fusions can

be expressed and regulated appropriately in the bacterium. This result based on fluorescence was supported by the α-GFP Western Blot, which showed modulation specific GFP transgene expression (Figure 9). For example, if the ratio of *cis*- to *trans*-acting factors were insufficient to adequately repress modulation-specific transgene expression a band would be evident in the non-modulated control lane of BP338 (pGB5-6). No such band was seen. Moreover, GFP over-expression was not traumatic for the organism, as growth curves for GFP-expressing bacteria were similar to those with no expression.

Another advantage over conventional transposon fusions was the ease of selection and versatility of GFP as a reporter for monitoring gene expression. Typically transposon-fusions rely on *lacZ* or *phoA* activity detection, which require that bacteria be tested for enzyme activity. GFP-expressing bacteria require no such treatment. Even while pre-selecting for GFP-expressing BP338 clones with a fluorescence microscope, the results are less ambiguous than for monitoring β–galactosidase activity for conventional Tn*lacZ* fusions. This is probably due to the sensitivity of β–galactosidase activity. In the DFI *bvg*-repressed gene screen described in this study, the great majority (98%; n=2000) of clones in the expression library did not express GFP and were not detectable by fluorescence microscopy. This is in contrast to a similar-sized screen for *bvg*-activated candidates where only a minority (3%) did not express β-galactosidase, as monitored by enzymatic detection (Weiss *et al.*, 1989). Background GFP reporter activity was not evident in BP338 clones harbouring promoter fusions in pGB5 and thus low-level induction was reproducible. In addition, the

quantification of GFP expression with a calibrated flow cytometer removes subjectivity associated with preparing samples for β-galactosidase or alkaline phosphatase colorimetric assays, due to human error.

In comparison with a previous transposon-based (Tn*phoA*) screen for candidate *bvg*-repressed genes done by Knapp *et al.* (1988), it is curious that the DFI screen yielded different proportions of candidate genes, even though similar numbers were screened. The DFI strategy was able to identify a larger proportion of potential *bvg*-repressed genes, thereby making the screen highly efficient. However, the GFP based screen was unable to distinguish, as the Tn*phoA* screen was, between proteins exported to the periplasm or expressed in general. Approximately 22% (n=40), of promoter traps were modulation-specific in the GFP-based screen described in this thesis compared to only 5% (n=99) of those captured by transposon insertion in the original *bvg*-repressed gene screen (Knapp and Mekalanos, 1988). The reasons for the discrepancy are not clear. The higher number of candidates identified by the promoter trapping DFI screen suggests that transposon insertion may, through mutation, inactivate modulation-specific genes that are also essential, thereby eliminating those clones from the mutant pool. This may be the case for genes involved in growth and metabolism. This may be likely given that two of the novel *bvg*-repressed genes identified are likely metabolic enzymes. It may also be that genes with less than two-fold effect cannot be reproducibly analyzed by colorimetric means since background levels are higher than for GFP expression and may be eliminated from the transposon-based screens. This could also account for the higher

number of colonies that were "on" in the transposon-based screen (n=99 *vs.* 40). Weiss' finding that 97% of Tn5*lacZ* insertions yielded productive fusions (as monitored by expression of β–galactosidase following insertion of the coding region of β-galactosidase, *lacZ*) in a *bvg*-activated gene screen, even though the maximal predicted value of 50% corroborates this. In addition, the use multi-copy number plasmids may elevate an otherwise weak signal and thus allow for selection. It is also possible, however, that the size of fragments chosen for library construction in the DFI screen (0.5-1 Kb) might generate artificial *cis*-acting sites resulting from the cloning of BP338 coding regions upstream of GFP. This is perhaps due to similarities between *bona fide* promoter sequences and artificial promoters created by coding regions. The cloning of artificial *cis*-acting sites may generate productive GFP fusions that are not driven by *bona fide* promoter elements. This is a possibility for at least the identified clone pGB5-P9. Another possibility is that extended regulatory domains may be missed by limiting the promoter size to 0.5 Kb as some promoters have upstream repressor or activator binding sites which may enhance or repress expression. Although these are legitimate concerns for developing the screen strategy, the prototypic *bvg*-repressed gene, *vrg6* requires less than 500 bp upstream of GFP and only the BvgR binding site to reconstitute modulation-specific expression. Therefore the initial size choice was valid for identifying novel *bvg*-repressed genes in *B. pertussis.*

In general, the DFI screen was productive in identifying novel modulation-specific genes with varying kinetics of induction. Two types of genes were

identified- those under BvgAS control and those that were modulation-specific by an unknown mechanism, that is they were able to respond to nictonic acid and magnesium sulfate by a mechanism that did not seem to involve the BvgAS regulon. The DFI screen identified clones with a range of GFP induction on agar between 1.5-and 4-fold, over a three-day time course, and mean fluorescence values below 30U. Low induction and expression levels are consistent with the majority of previously characterized *bvg*-repressed genes which were induced between three and five fold (*vrg6*::Tn*phoA*, *vrg18*::Tn*phoA*, *vrg24*::Tn*phoA*) with only one identified mutant capable of high induction (*vrg73*::Tn*phoA*) at 25 fold (Beattie *et al.*, 1993). Although the typical range of *bvg*-repressed gene induction is three to five-fold, the DFI screen was able to select for even lower expression and induction mutants (less than three fold, below 30) because the background expression of GFP is negligible compared to other reporters (β-galactosoidase, alkaline phosphatase). This is beneficial since any gene that is up-regulated in response to modulators may provide important clues to the actual signals and niche that trigger this phenotypic switch in *B. pertussis.* Indeed one *bvg*-regulated clone pGB5-P2 had induction of only 1.5 fold at its peak on BGA with modulators, as did the modulation-specific clone pGB5-P7 (Figures 13A and 15A. Despite low GFP expression in all agar-grown modulation-specific clones identified, including the control strain pGB5-6, all bacterial colonies were readily identified as positive for GFP expression by fluorescence microscopy. This result was corroborated by flow cytometeric analysis, which produced mean fluorescence

values at least 5× above background (e.g. BP338 pGB5, pGB5-3) for all clones tested.

The screen had the added advantage that bacteria could be grown in a variety of growth conditions and tested for GFP expression immediately. This made it possible to identify the dramatic differences between agar- and broth-grown cultures that may illuminate alternate environmental regulation of the modulatory response. All modulation-specific clones had diminished GFP expression when grown on agar, yet those clones controlled by the BvgAS regulon were most noticeably affected. Clones pGB5-P2, pGB5-P9 and pGB5-P10 had between 4-10× higher absolute levels of GFP and upwards of two-fold higher DFI levels in SSB with modulators. The control strain pGB5-6 with a minimal *vrg6* promoter also had 2× higher GFP levels, but similar DFI values. This was not a general phenomenon as the constitutive promoter pGB5-P1 expressed similar levels of GFP in agar and broth. The reason for this result is not clear; however, a clue to the regulation of these clones in SSB is evident in their marked expression differences in high osmolarity broth. Clones that were under the regulatory control of BvgAS, as confirmed by constitutive GFP expression in the BP907 background, had diminished GFP expression when grown in SSB+10% sucrose, despite high levels of GFP expression in regular SSB. GFP expression was all but abolished in these clones, whereas clones that exhibited modulation-specific induction in agar, but did not appear to be BvgAS regulated, still had high levels of GFP expression (50-100U). In fact, in the clone pGB5-P7, modulation-specific expression was exaggerated by two-fold (from 1.5

fold-3 fold DFI) in high osmolarity broth, indicating that this condition was favorable for this modulation-specific induction. Thus it appears that only those modulation-specific clones that are BvgAS controlled *respond* similarly to adverse high osmolarity conditions and shut off reporter gene expression.

It is intriguing that only the clones that appeared to be *bona fide bvg*-repressed genes were repressed by high osmolarity growth conditions. This suggests that either BvgAS is sensing osmolarity directly or possibly there is another input into *bvg*-repressed gene regulation than previously identified. It is well established that BvgAS responds to temperature, nicotinic acid, sulfate anions and B-vitamin derivatives but osmolarity has never been described. In many gram-negative bacterial species the two-component regulator OmpR-EnvZ controls the expression of numerous genes, including the porins OmpC and OmpF, in response to changes in osmolarity via a classic phosphorelay cascade (Graeme-Cook *et al.*, 1989; Puente *et al.*, 1991). In addition, several aspects of virulence gene expression are under osmoregulatory control, including invasion in *Salmonella typhimurium* (Bajaj *et al.*, 1995). It is possible, therefore, that *B. pertussis* may be using a homologous system for osmoregulation. Indeed OmpR homologues are present in the *Bordetellae* including *B. bronchispetica* and *B. pertussis;* however, in all *B. pertussis* strains tested, the *envZ* sensor locus is silent (D. Maskel, personal communication). If the response regulator is active in the organism then it must be acting with an alternate sensor molecule. Since cross-talk between two component regulators is a well documented phenomenon, it is possible that *trans*-activation is occurring through an alternate

environmental sensor (Arthur *et al.*, 1999; Volz, 1995). One logical possibility is that the *trans*-activator is BvgS. This complex sensor molecule has several domains, which may be important for *trans*-activation of molecules other than BvgA. It is possible that this could offer another level of *bvg*-repressed gene regulatory control. Recent findings by Stenson and Peppler (manuscript in preparation) corroborate this conclusion. It was shown that an *ompR* transposon mutation greatly diminished the expression of two *bvg*-repressed antigens VraA and VraB, such that specific antibodies could detect only faint expression under modulatory growth conditions. It was further demonstrated that *bvg*-activated genes were not affected by this mutation, which is consistent with the claim that osmolarity conditions specifically hinder *bvg*-repressed gene expression. It is unclear whether the effects of this additional regulator would be directly at the level of transcription; however, if other OmpR regulons are any indication, then *bvg*-repressed genes are likely to have upstream *cis*-acting sites that can act as activation and repression domains. In other organisms, like *Escherichia coli,* OmpR regulated genes including *omp*F are differentially regulated by the amount of phosphorylated OmpR present in the cell. High affinity sites that bind low levels of phosphorylated OmpR favor transcriptional activation, whereas low affinity sites, which require high levels of phosphorylated OmpR, favor repression (Head *et al.*, 1998). Such a system in *B. pertussis* may act to ensure that *bvg*-repressed genes are off under conditions of high osmolarity when high levels of active OmpR are present in the cell. Evidence from Stenson and Peppler that OmpR null mutants do not express *bvg*-repressed genes corroborates this

hypothesis; however, many possibilities exist for both direct and indirect osmo-regulation.

Partial sequencing of the cloned fragments allowed the identification of all the inserts on unfinished contigs in the *B. pertussis* sequencing project Blast database. The contigs were then searched using the BLASTX algorithm to identify possible downstream open reading frames (ORFs) and upstream promoter sequences in the non-redundant (nr) database. All clones had potential homolgues in other organisms, although for some the matches were stronger than for others. For example, clones pGB5-P2, pGB5-P5 and pGB5-P10 had high sequence matches over extended protein regions (greater than 59% identity) and significantly low P values. Clones pGB5-P7 and pGB5-P9 were more problematic in that they shared high amino acid identity, but only a limited region was available for the search, due to the incomplete genome sequence. Clone pGB5-P9 had the added problem that it seemed to be comprised entirely of an ORF with no known promoter. It is possible that the proteins identified in the latter two clones are legitimate, however, the answer must await the completion of the *B. pertussis* sequencing project.

All novel clones identified shared homology with metabolic, structural genes and the response regulator, YuxN, in other species. Although no classical virulence genes were identified, these gene products represent the broader category of some well-understood adaptive responses to environmental stresses, which may illuminate the niche where such responses are required. For example, the clone pGB5-P2 shares high homology with a hydroxyacid

dehydrogenase from *Arabidopsis* and many bacterial species. Dehydrogenases, which catalyze the reduction-oxidation (REDOX) reaction involving the transfer of hydrogen from one substrate to another, are usually involved in respiration. However, hydroxyacid dehydrogenases, like D-lactate dehydrogenase, have been implicated in peptidoglycan synthesis, which is critical for the turgor of the bacterial cell wall (Ferain *et al.*, 1996). Thus, a seemingly insignificant metabolic gene may have major repercussions for the cell in terms of a stress response. The ability to maintain the cell wall is critical for bacterial survival and indeed may be influenced by numerous overlapping stress response pathways, including those that may respond to osmolarity and modulation. Perhaps the bacteria require heightened or modified peptidoglycan synthesis during modulation to combat the stress of this environment. This potentially illustrates the complexity of the modulatory niche as an environment that may require alternate cell wall precursors in order to insure bacterial survival.

Another metabolic enzyme was identified as the bacterial homologue of clone pGB5-P5. The clone encodes the promoter and partial ORF for a putative inosine 5'-monophosphate dehydrogenase (IMPDH, *guaB*), the rate limiting enzyme for *de novo* guanine nucleotide biosynthesis. This is part of the larger purine metabolism pathway, which contributes to nucleic acid biosynthesis (Weber, 1983). As a metabolic process, purine metabolism is imperative for bacterial survival; however, IMPDH has also been shown to be critical for microbial pathogenesis (Fields *et al.*, 1986; Mahan *et al.*, 1993; McFarland and Stocker, 1987; Noriega *et al.*, 1996). Intriguingly, *Salmonella guaB* mutants are

impaired in intracellular survival compared to their wild-type counterparts. This may be because intracellular purine levels are limiting for bacterial growth in the human host. Given that this gene product was specifically induced in agar under modulatory growth conditions indicates that the modulatory environment may at some time be purine-limiting. It is intriguing to speculate that modulation can occur inside human cells and this is the first gene with known intracellular function that corroborates the speculation.

Another stress response protein identified in the screen is the potential bacterial cardiolipin synthase, clone pGB5-P9. Since the entire contig contained only a small section of the captured protein it is difficult to determine whether the product is a homologue, however, the similar protein motifs may illustrate the function of the protein nonetheless. No cardiolipin synthase gene product has been discovered in *B. pertussis* to date. Cardiolipin synthases in general are involved in cell lipid metabolism, which is critical for maintaining cell wall integrity. (Hiraoka *et al.*, 1993; Ohta *et al.*, 1985; Tropp, 1997) In *E. coli*, these gene products are induced during stationary growth when the bacteria are not actively dividing. They play a critical role in bacterial survival and null mutants lose viability after prolonged incubation (Hiraoka *et al.*, 1993). This is perhaps due to a decrease in cell wall integrity caused by the instability of alternate cell wall precursors during prolonged growth. Indeed, in previous studies, cardiolipin was found to be the most stable phospholipid during *E. coli* growth. Since expression of this homologue in *E.coli* is increased in stationary phase it implies that the product is required during a metabolically less active period. This fits with the

hypothesis that *B. pertussis* invades alveolar cells and enters a quiescent stage which would require a stable cell wall structure to insure long term survival. Even if pGB5-P9 turns out not to be the *bona fide* homologue of cardiolipin synthase, it may well be a structural cell wall component based on similar protein motifs.

Response regulators have an integral role in coordinating gene expression during bacterial growth and infection. Clone pGB5-P7 contained the partial regulatory region with homology to a putative transcriptional regulator with a helix-turn-helix motif from *Bacillus subtilis*. It may also be a transciptional regulator in *B. pertussis* and aid in the activation and repression of gene products involved in the modulatory cascade. This clone was interesting in that it had reciprocal phenotypes when grown in agar or broth. In agar it behaved as a modulation-specific determinant but in broth it was greatly induced without modulators (*bvg*-activated expression). This was corroborated with growth in high osmolarity broth, where expression was similar to that in agar. Such a response pattern may be indicative of a link between osmo-regulation and modulation. The expression seemed at least in part to be BvgAS-dependent as the BP907 strain had 4-fold induction when grown on BGA with modulators. This was increased from the 1.5-fold induction in the wt BP338 background, which indicates that the BvgAS regulon may be somewhat repressive and moreover the modulators seem to be working through an alternate cascade. In addition, the broth data would suggest that the BvgAS regulon may be involved at least in part in the regulation of this clone as the BP907 strain had constitutive GFP expression. Taken together, this implies that modulating signals may induce an

alternate regulon in *B. pertussis*. Given the GFP induction kinetics for this clone, such a regulon may respond to high osmolarity and modulating signals and be repressed in part by BvgAS. The opposite pattern evident during broth growth adds complexity to the system. Traditional *bvg*-repressed genes typically were repressed in high osmolarity (BGA, SSB+ 10% sucrose) growth conditions and induced in low osmolarity growth conditions (SSB), but never change from *bvg*-repressed to *bvg*-activated genes. This new modulatory response in clone pGB5-P7 is different from any known *bvg*-repressed gene response in *B. pertussis* and may be an important bridge between osmolarity and novel modulatory cascades. If it is indeed a *bona fide* response regulator, then it would be critical to identify what gene products it may regulate to fully understand the nature of this peculiar, novel response.

It is always encouraging when a novel screen is successful in finding previously missed gene targets that can be the subject of further study and elucidation. It is also beneficial when such a screen uncovers previously identified gene products, which act as an internal control for the effectiveness of the novel approach. Clone pGB5-P10 turned out to be the previously characterized *vrg6*. Although the BvgR binding site was present in the captured promoter, the clone itself was twice the size of the control strains pGB5-3 and pGB5-6 and had more upstream sequence. This was undoubtedly responsible for the differences in the GFP induction kinetics and expression of the captured and constructed clones. Although the clones behaved similarly in agar, there was a three-fold induction difference in broth between the clones. It would be

interesting to determine which upstream sites are responsible for this broth-specific induction and what additional response regulators are responsible for this phenomenon.

Although only three of the five novel modulation-specific clones behaved as *bona fide bvg*-repressed genes, all but one (pGB5-P5) had a putative BvgR binding site (refer to Figures 22-25). Based on the four previously identified *bvg*-repressed genes and the newly identified clones, the consensus was updated to better reflect the heterology of the site. It is intriguing to note that certain anchor residues are common to all the clones, which are likely to be critical for contact with BvgR. The updated consensus is presented with IUPAC notation in Figure 26, (refer to figure 8 for comparison).

Figure 26: Updated BvgR consensus based on the new modulation-specific determinants identified in this study. Standard IUPAC notation is used. Refer to Figure 8 for comparison to the previously published BvgR binding site consensus

5. Conclusions and Future Perspectives

This study has contributed to the understanding of the BvgAS regulon in *Bordetella pertussis* by uncovering four novel modulation-specific genes through a series of promoter library fusions in a GFP-expressing plasmid system. With the aid of flow cytometry, induction kinetics in various growth conditions could be assessed in a quantitative manner for an entire bacterial population on an individual (per bacterium) basis. Although the various clones behaved differently in the analysis, it was clear that some commonality existed amongst the isolates. Without fail, all the clones that were *bona fide bvg*-repressed genes reacted adversely to high osmolarity growth conditions and showed diminished modulation-specific GFP induction. This adds a level of complexity to the classical understanding of the modulatory cascade. Although the BvgAS regulon has been known to respond to various stimuli, osmolarity has never before been associated with modulation. This new clue enhances previous knowledge of the modulatory response and establishes a potential new link between osmo-regulation and modulation. This may illustrate how *in vivo* modulatory signals are triggered. In order to confirm the connection between osmo-regulation and modulation, the next critical experiments for the *bvg*-repressed clones that showed inhibited GFP expression in high osmolarity broth would be an analysis of induction kinetics in an OmpR null background. If the BvgAS-specific expression is also osmo-dependent in these clones, then an OmpR mutation should cause a dramatic decrease in GFP expression. Indeed it has been shown

that for two previously identified *bvg*-repressed antigens, expression is diminished in an OmpR mutant background (Stenson and Peppler, manuscript in preparation).

It is clear from this study that alternate regulators may impact BvgAS regulation and the modulatory cascade. In addition to osmolarity having an impact on expression, it became evident that some clones (pGB5-P5 and pGB5-P7) were at least in part modulation-specific without BvgAS. The observation that these clones can respond to modulatory signals, namely nicotinic acid and magnesium sulfate, in the absence of a functional BvgAS sensor illustrates that an alternate sensor must be responsible for some degree of modulation-specific induction in *B. pertussis*. This is a relatively unexplored area, since it was previously thought that *bvg* was the main virulence regulon in the organism, however, the findings in this study illustrate the potential for other inputs into the modulatory cascade. It would be interesting to screen for more modulation-specific genes that are not in the *bvg* regulon. This can be achieved by performing the same screen with the BP907 background to look for clones that still respond to modulators. These clones would not have a functional BvgA and therefore any modulation-specific induction would likely be due to another signaling pathway. Such a screen could potentially add tremendous insights to our understanding of how modulation occurs in the organism.

In order to complement the promoter-trapping method, which yields candidate promoters that are regulated by modulation, a more in depth genomic approach should simultaneously be explored. With newly developed microarray

technology, it should be possible to identify upregulated targets from any strain and under any growth condition. This new technology offers an advantage over promoter trapping in that a comprehensive analysis can be undertaken at once for any growth condition (Arfin *et al.*, 2000; Debouck and Goodfellow, 1999; Edman *et al.*, 2000). The microarray assay is made powerful by allowing for the analysis of reverse-transcribed cDNA products of bulk-purified mRNA, that is hybridized to an array containing fragments of all known genes in a given organism. The readout is made possible by tagging the nucleotide tri-phosphates (dNTPs) used in the reverse-transcription reaction with fluorochromes that can be detected by laser excitation. Since the hybridization of the cDNA to target DNA on the microarray is directed by homology, the readout of fluorescence is directly proportional to the amount of initial mRNA present in the sample. Thus strongly induced genes give high fluorescence values whereas repressed genes will have low values. The ideal way to identify all the *bvg*-repressed factors at once would involve comparing the gene-array expression profiles, as a function of mRNA levels, for modulated and non-modulated BP338 isolates as well as the for *bvg*A deletion mutant BP907. The profiles for the non-modulated and modulated strains can then be subtracted to yield *bvg*-repressed candidates. Such an approach can be repeated with OmpR mutants to identify what genes are osmo-regulated. Any microarray profile can be compared to identify genes under common control. This approach has enormous potential, but is not problem-free, as reproducibility amongst probed microarrays is a concern. It may not be possible to rely on two-fold induction data, as "chip-sets" can vary

by as much as much as 30% or more depending on how the arrays are made. This is a problem for genes that are typically induced between two and four-fold. In addition, if mRNA levels do not adequately illustrate the protein activity level, as for proteins that are post-translationally modified before they are active, the microarrays will not be indicative of the protein expression kinetics. As well, such an approach does output the profile of an entire population and not an individual bacterium. Nevertheless, an attempt should be made to screen a *B. pertusis* microarray with mRNA isolated from non-modulated and modulated bacteria to determine if at least known virulence and modulation-specific determinants can be identified using this approach. If this is successful, the potential exists to rapidly identify many of the genes in the BvgAS regulon. This would greatly add to our understanding of *B. pertussis* pathogenesis.

Most genes identified in this study have homolgues in other species that are known to be essential metabolic and stress response proteins. Individually, their expression is known to be up-regulated in a variety of environments: stationary phase, intracellular growth and environments of increased cell wall stress. It has been hypothesized that modulation in *B. pertussis* may occur in an intracellular niche in order to avoid immune detection and possibly to establish a long term carrier state. In such an environment it is likely that the bacterium may enter a metabolically less-active stage and be exposed to cell wall stress. Although the identification of these genes as modulation-specific determinants in no way establishes proof for this speculatory niche, it nonetheless is an important clue, which should be explored. It is important to establish whether these genes

are actually up-regulated *in vivo* and this will require that GFP expression be monitored during a tissue-culture invasion assay. Such an approach has been successful for monitoring intracellular gene expression in *S. typhimiurium,* however it is less straightforward for *B. pertussis* (Valdivia and Falkow, 1996). Since *bvg*-repressed gene expression is faint and the bacteria are small in size, it may be difficult to get an accurate intracellular GFP read-out even for FACS-sorted tissue culture cells. It is important, nonetheless, to assess whether these products are truly expressed since it is possible to capture a promoter element that is functional but isn't actually productive *in vivo*. This is important since *B. pertussis* has many pseudogenes which are encoded but not expressed due to premature stop codons or promoter mutations, including the flagella operon (Akerley and Miller, 1993). One way to assess expression is to check for protein expression on a Western Blot. Since homologues exist in other prokaryotes, it may be possible to obtain some cross-reactive antibodies to probe the Westerns.

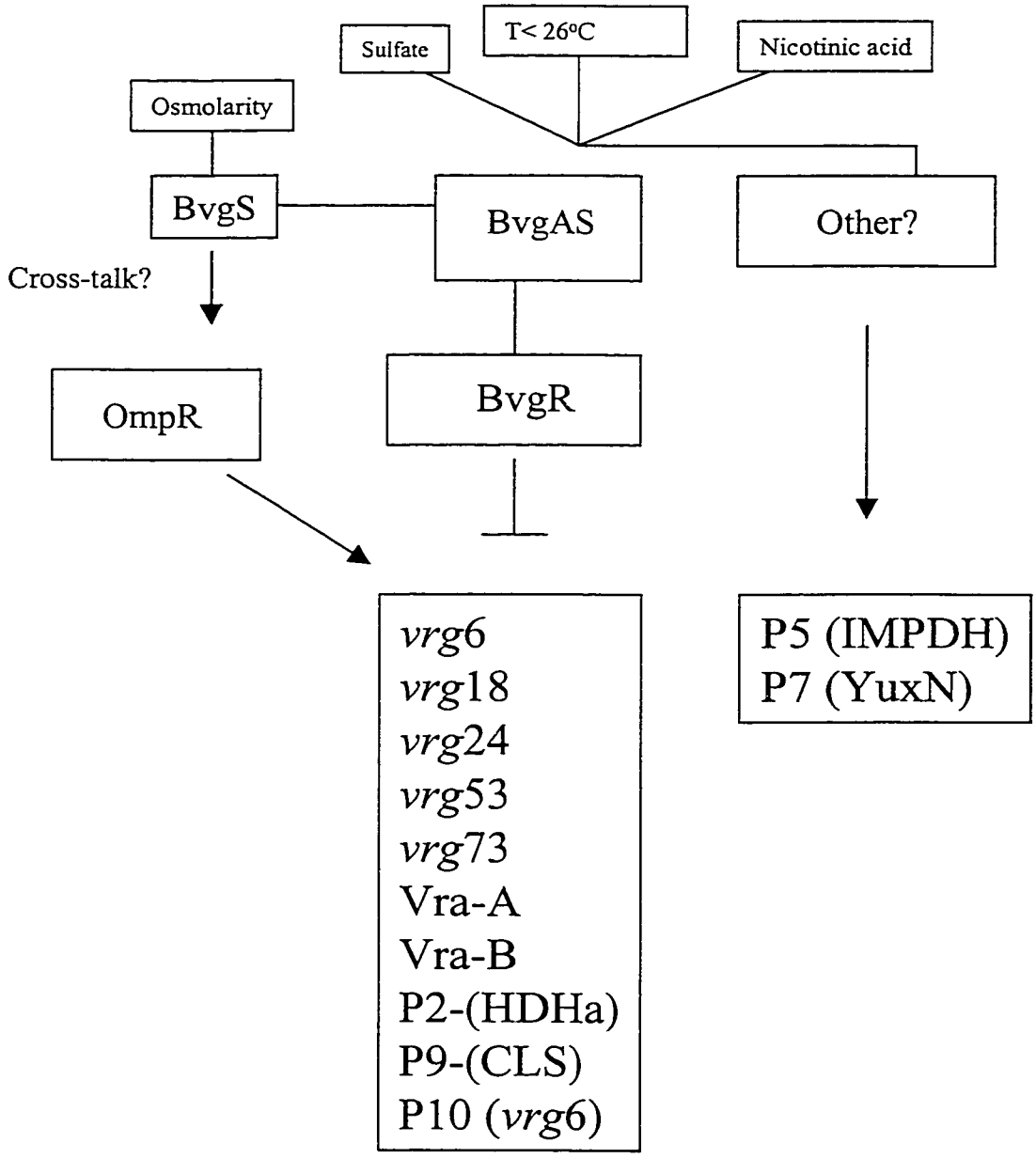
In conclusion, this study has identified four novel gene products with homologues in other prokaryotes that may be involved in metabolism, cell structure and signal transduction. Although no classical virulence determinants were identified, these genes are part of general stress response pathways that may aid survival for the bacterium in a yet-unknown developmentally regulated niche. It is clear that osmolarity impacts *bvg*-regulated gene expression and the possibility exists for cross-talk between the BvgS sensor and the OmpR response regulator. In addition, this study has uncovered two genes P5 and P7 that are modulation-specific by an unknown mechanism that is responsive to the

classical modulators, magnesium sulfate and nicotinic acid. A model for the multiple signal inputs that culminate in *B. pertussis* modulation is presented in Figure 27.

There is much speculation in the field as to whether there is an intracellular niche during the later stages of infection, or during an "asymptomatic" carrier state, where the bacterium would avoid host detection by down-regulating virulence determinants through modulation. Although this hypothesis was not directly tested in this study, several intriguing discoveries were made. Namely, some genes identified in the promoter-trap screen are consistent with expression in an intracellular environment, like the IMPDH homologue, pGB5-P5 which is known to be required in other organisms for invasion and survival (McFarland and Stocker, 1987; Noriega *et al.*, 1996). The two stress response genes that were homologous to cloned sequences of pGB5-P2 and pGB5-P9, hydroxyacid dehydrogenase and cardiolipin synthase, respectively, do not specifically illustrate an intracellular niche for expression, but they are consistent with the modulatory niche being a somewhat hostile environment. The last homologue, pGB5-P7, a response regulator type protein, is interesting since that alternate regulators may be involved in *B. pertussis* modulation and may respond by activating other regulatory cascades.

Further study is needed to understand the complex host-pathogen interactions between *B. pertussis* and its human-only niche. In light of these findings it is imperative to identify as many more modulatory factors as possible

Figure 27: Model for multiple sensory inputs for *B. pertussis* modulation



to help illustrate the role of this elusive set of proteins. Recent findings in our laboratory have implicated OmpR as a major contributor to *bvg*-repressed gene expression, and further, that *ompR* mutants are impaired for survival in a tissue culture assay. This study has corroborated that *bvg*-regulated factors are repressed by conditions of high osmolarity. Taken together, these results suggest a role for alternate signaling pathways for modulatory control in *B. pertussis*, which will undoubtedly aid in the elucidation of the true *in vivo* modulatory signal. Indeed the genes identified here may illustrate the true nature of the modulatory niche, as previously identified *bvg*-repressed genes have failed to have homologues in other pathogenic bacteria. Only as more evidence is uncovered about the nature of *bvg*-repressed gene regulation and expression in *B. pertussis* will we truly begin to understand the purpose of the phenomenon first described as "antigenic modulation" by Lacey in 1960.

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7. Appendix

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Fluorescent Labels Influence Phagocytosis of *Bordetella pertussis* by Human Neutrophils

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To explore the role of neutrophil phagocytosis in host defense against *Bordetella pertussis*, bacteria were labeled extrinsically with fluorescein isothiocyanate (FITC) or genetically with green fluorescent protein (GFP) and incubated with adherent human neutrophils in the presence or absence of heat-inactivated human immune serum. In the absence of antibodies, FITC-labeled bacteria were located primarily on the surface of the neutrophils with few bacteria ingested. However, after opsonization, about seven times more bacteria were located intracellularly, indicating that antibodies promoted phagocytosis. In contrast, bacteria labeled intrinsically with GFP were not efficiently phagocytosed even in the presence of opsonizing antibodies, suggesting that FITC interfered with a bacterial defense. Because FITC covalently modifies proteins and could affect their function, we tested the effect of FITC on adenylate cyclase toxin activity, an important extracellular virulence factor. FITC-labeled bacteria had fivefold-less adenylate cyclase toxin activity than did unlabeled wild-type bacteria or GFP-expressing bacteria, suggesting that FITC compromised adenylate cyclase toxin activity. These data demonstrated that at least one extracellular virulence factor was affected by FITC labeling and that GFP is a more appropriate label for *B. pertussis*.

Bordetella pertussis is the obligate human pathogen that causes whooping cough. This organism produces a battery of virulence factors such as pertactin, BrkA, filamentous hemagglutinin (FHA), fimbriae, adenylate cyclase toxin, tracheal cytotoxin, pertussis toxin, and dermonecrotic toxin (20). These factors are either adhesins or toxins that mediate colonization of respiratory tract epithelial cells or resistance to host defenses.

Immunity to *B. pertussis* is mediated through natural infection or vaccination with whole-cell or acellular vaccines. The mechanism of protection, however, is not completely understood (11, 16). Neutralization of pertussis toxin and blocking of bacterial attachment to ciliated cells are likely to be important in immunity, but opsonization, phagocytosis, and bacterial killing also may play a role in protection. We are interested in studying the role of human antibodies against *B. pertussis* virulence factors in promoting opsonization and phagocytosis.

To measure phagocytosis of *B. pertussis* by human neutrophils, we needed to develop an assay that distinguished intracellular from extracellular bacteria. Fluorescein isothiocyanate (FITC) labeling of microorganisms has been used extensively as a convenient way to visualize bacteria interacting with mammalian cells (4, 8, 9). Basically, bacteria labeled with FITC are incubated with the mammalian cells of interest and then counterstained with ethidium bromide; intracellular FITC-labeled bacteria resist staining with ethidium bromide and remain green, but extracellular ethidium bromide-labeled bacteria appear orange by fluorescence microscopy (4, 8, 9). FITC covalently binds primary amines of amino acids present on the N terminus of proteins and on lysine residues. It labels only amines in the free base (uncharged) state, and a high pH (>8) is used to increase the efficiency of FITC labeling. We were concerned that FITC labeling could give misleading results by either modifying proteins critical to the function of a biologically important protein or affecting the viability of the bacteria. In this study, we compared FITC and green fluorescent protein (GFP) labeling of live *B. pertussis* to determine whether either labeling procedure had an effect on phagocytosis of opsonized and nonopsonized bacteria.

Labeling bacteria with FITC. Bacteria were labeled by a modification of the procedure of Hazenbos et al. (8). Bacteria from overnight cultures on Bordet-Gengou agar (BGA; Difco, Detroit, Mich.) were harvested with Dacron swabs (Fisher, Pittsburgh, Pa.), suspended into phosphate-buffered saline, and adjusted to an A_{600} of 1 or about 2×10^9 bacteria/ml. Bacteria (2×10^8) were transferred to a microcentrifuge tube, pelleted, and suspended in 1 ml of FITC (Sigma, St. Louis, Mo.) (0.5 mg/ml) in 50 mM sodium carbonate–100 mM sodium chloride at various pH values. Bacteria were incubated for 20 min at room temperature, washed three times in 1 ml of HBSA (Hanks' buffer [Biowhittaker, Walkersville, Md.] supplemented with 0.25% bovine serum albumin [Sigma] and 2 mM HEPES [Calbiochem, San Diego, Calif.]) at 34,500× *g* for 10 min at 4°C, and then suspended in 100μl of HBSA.

We examined the effect of pH on FITC labeling and on fluorescence intensity and viability. Bacteria labeled at pH 7.7, 8, 8.5, 9, and 10.5 were easily visualized by fluorescence microscopy. Bacterial survival was determined by plating on BGA; no loss in viability was observed, except at pH 10.5, where <1% survived. The bacteria were labeled at pH 8 for all other experiments.

The effects of ethidium bromide staining also were examined. Bacteria incubated for 5 or 30 min with ethidium bromide (50 μg/ml in Hanks' buffer) appeared orange with a bright central stain, suggesting that the stain penetrates the membranes and binds to the DNA of *B. pertussis*. Incubation with

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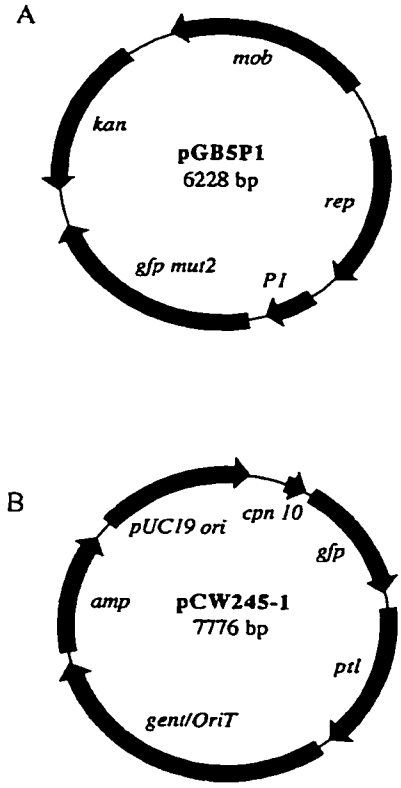

FIG. 1. Constructs for GFP expression. (A) pGB5P1. The *gfp*-mutant 2 gene (2) was cloned as a *Bam*HI-*Eco*RI restriction fragment into the pBBR1MCS-2 vector (15). A *Sau*3A restriction fragment that encodes a constitutive *B. pertussis* promoter was cloned upstream to control *gfp* expression. (B) pCW245-1. Nucleotides 1 to 251 from the *B. pertussis cpn 10* (5) promoter were amplified by PCR, and the product was digested with *Pst*I and *Hae*III and then cloned into pGFPuv to control *gfp* expression, generating pCW211-6. A *Pst*I restriction fragment (nucleotides 11810 to 13025) from the end of the *ptl* operon was cloned into pUW2139 [pBluescript SK(+) containing *gent/oriT*], and the resulting construct, pCW204-1, was digested with *Apa*I and ligated with pCW211-6 to generate pCW245-1. Plasmid CW245-1 was introduced into bacteria by triparental mating as previously described (19), and transformants were selected on BGA, nalidixic acid, and gentamicin (30 μg/ml). Abbreviations: *mob*, mobilizable gene; *rep*, plasmid replication; *gfp mut2*, green fluorescent protein mutant 2; *P1*, *B. pertussis* constitutive promoter; *kan*, kanamycin; *cpn 10*, chaperonin 10 (*B. pertussis* GroES homologue); *ptl*, pertussis toxin liberation; *gent/oriT*, gentamicin/origin of transfer; *amp*, ampicillin.

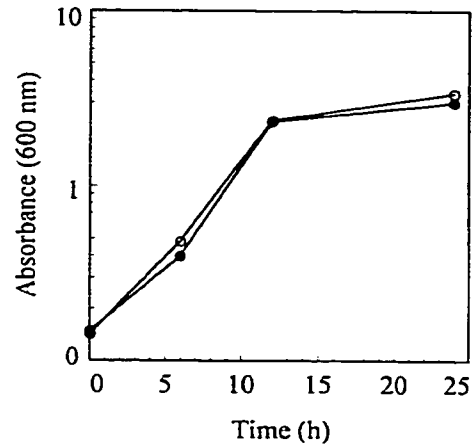

FIG. 2. Effect of GFP on growth. Bacteria from overnight BGA were suspended into SS broth to an A_{600} of 0.1. Five milliliters of the BP338 and BP338(pGB5P1) suspensions was distributed to BGA containing nalidixic acid and nalidixic acid with kanamycin, respectively, and cultures were incubated at 37°C. Bacteria were harvested at 6, 12, and 24 h and washed, and the absorbance was measured. ○, BP338; ●, BP338(pGB5P1).

ethidium bromide affected bacterial viability; <10% of the wild-type bacteria survived both the 5- and the 30-min treatments.

Labeling bacteria with GFP. An alternative method, cytoplasmic expression of GFP, has been used to label bacteria to study bacterial interactions with mammalian cells (3, 18). We used two constructs, pGB5P1 and pCW245-1 (Fig. 1), for the cytoplasmic expression of GFP. Plasmid pGB5P1 was introduced into wild-type BP338 (22) by electroporation by a modification of the method of Zealey et al. (25). Briefly, bacteria were grown in 500 ml of Stainer-Scholte (SS) broth at 37°C for 72 h with rotation. Bacteria were harvested (11,350 × *g*), washed twice in sterile distilled water and once in 272 mM sucrose–15% glycerol (SG), suspended in 10 ml of SG, and stored at −80°C in 600-μl aliquots. Plasmid pGB5P1 DNA (10 μg) was added to competent bacteria, pulsed at 2.5 kV (Bio-Rad *Escherichia coli* pulser) with an electrode gap of 0.2 cm, transferred to 5 ml of SS broth, and incubated at 37°C for 1 h with rotation. The culture was divided among five microcentrifuge tubes, pelleted at 5,160× *g* for 5 min, suspended in 100 μl of SS broth, and plated onto BGA and kanamycin (50 μg/ml) and nalidixic acid (30 μg/ml) to select for resistant electroporants. Plasmid pCW245-1 was introduced into the chromosome of wild-type BP338 and adenylate cyclase toxin mutant BP348 (22) by triparental mating as previously described (19) with the pertussis toxin homologous region, resulting in strains BP338 *ptl*::pCW245-1 and BP348 *ptl*::pCW245-1, respectively. Western blot analysis with an S1 monoclonal antibody, C3X4 (14), has shown that recombination at the end of the operon does not affect pertussis toxin expression (data not shown).

Expression of GFP did not affect bacterial growth. In Fig. 2, the growth rates of wild-type strain BP338 and strain BP338(pGB5P1) expressing GFP were identical. The expression of virulence factors was not affected by GFP expression; BP338(pGB5P1) was hemolytic and expressed pertussis toxin, lipopolysaccharide, pertactin, BrkA, and FHA at levels comparable to those for the parental strain by Western blotting or protein gel electrophoresis (data not shown). BP338 *ptl*::pCW245-1 also was similar to the wild type in growth rate and protein expression. Therefore, GFP does not seem to adversely affect bacterial growth or gene expression.

Phagocytosis assay. Human neutrophils were purified as previously described (10) and quantified on a hemacytometer. Neutrophils (5×10^5/well in 1 ml of HBSA) were permitted to adhere to round glass coverslips in 24-well plates for 1 h at 37°C in 5% CO_2.

To investigate the role of opsonization by antibodies in the absence of complement, serum sample 13 (24) was heat inactivated at 56°C for 30 min. This serum is a previously characterized serum sample from an individual with occupational exposure to *B. pertussis* and has antibodies to *B. pertussis* lipopolysaccharide as well as several surface-localized protein virulence factors.

Overnight BGA cultures of wild-type or GFP-expressing bacteria were harvested and labeled with FITC where indicated. Bacteria (3×10^6 in 30 μl) were transferred to microcentrifuge tubes and incubated with human immune serum (30

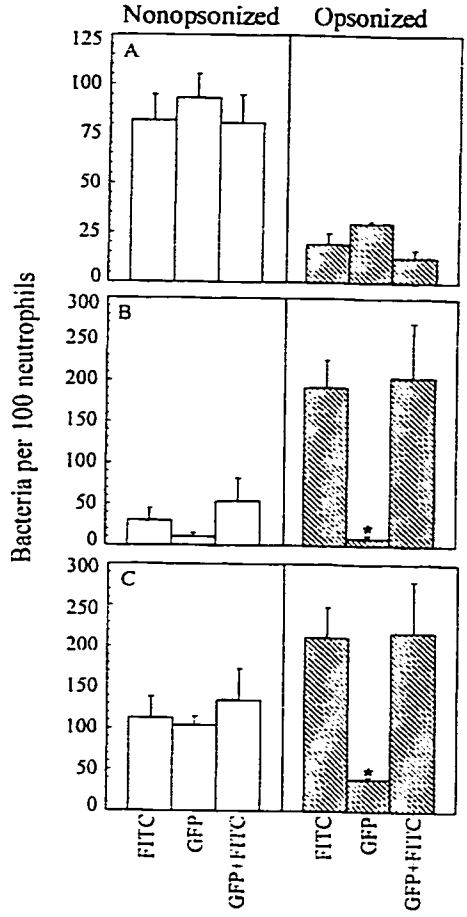

FIG. 3. Effect of labeling treatments on phagocytosis. One hundred consecutive neutrophils were counted by fluorescence microscopy. (A) Number of orange (extracellular adherent) bacteria per 100 neutrophils. (B) Number of green (intracellular, phagocytosed) bacteria per 100 neutrophils. (C) Total association; number of orange and green bacteria per 100 neutrophils. FITC, BP338 labeled with FITC. GFP, BP338(pGB5P1). GFP+FITC, BP338(pGB5P1) labeled with FITC. Data were analyzed by the Student *t* test. Each bar represents the mean (± standard error of the mean). *, significantly different from the FITC labeling treatment ($P < 0.05$).

μl) or HBSA buffer at 37°C for 15 min. Bacterial suspensions were adjusted to 400 μl with HBSA, added to 5×10^5 adherent neutrophils, and incubated at 37°C in 5% CO_2 for 1 h. The suspensions were aspirated, and the neutrophils were washed once with 1 ml of HBSA to remove unattached bacteria. To stain bound but not ingested bacteria, ethidium bromide (50 μg/ml in 1 ml of Hanks' buffer) was added for 5 min at room temperature and then removed by aspiration. Neutrophils were fixed and mounted as previously described (17). Phagocytosis was quantified by phase-contrast and fluorescence microscopy on a Zeiss microscope with a 09 filter set (wide band pass exciter, 450 to 490; long pass emission, 520 and above). Each assay was performed three times in duplicate.

Comparison of labeling treatments. The number of extracellular adherent bacteria was similar for both labeling conditions, about 80 bacteria attached per 100 neutrophils (Fig. 3A, open bars). Interestingly, fewer adherent bacteria were observed following opsonization (Fig. 3A, striped bars), suggesting that adhesin-mediated attachment (i.e., by FHA or pertactin) may be more efficient than Fc-mediated attachment.

Phagocytosis was also examined in the absence of antibodies; about 30 FITC-labeled BP338 bacteria and about 10 GFP-expressing bacteria per 100 neutrophils were phagocytosed (Fig. 3B, open bars). As a point of reference, this is only about 5 and 2% of the total bacterial inoculum, respectively. When FITC-labeled BP338 bacteria were opsonized with heat-inactivated human immune serum, six times more bacteria were phagocytosed (Fig. 3B, striped bars). However, unlike the FITC-labeled bacteria, opsonization with immune serum did not increase the efficiency of phagocytosis of the GFP-expressing bacteria. Total bacterial association is shown in Fig. 3C.

These results suggested that FITC labeling interfered with the ability of *B. pertussis* to evade phagocytosis. We tested this hypothesis by labeling GFP-expressing BP338 with FITC. The results with these bacteria were comparable to those with the FITC-labeled wild-type bacteria. This is most apparent when phagocytosis of the opsonized bacteria is compared (Fig. 3B, striped bars).

TABLE 1. Effect of labeling treatments on adenylate cyclase activity

Organism	pmol of cAMP/min/10^7 bacteria[b]
BP338	5,520 (±425)
BP338 plus FITC[a]	1,200 (±69)[c]
BP338 *ptl*::pCW245-1	6,840 (±408)
BP348 *ptl*::pCW245-1	0 (±4)[c]

[a] Bacteria were labeled with FITC at pH 8.
[b] Data in parentheses are standard errors of the means.
[c] Significantly different from BP338 ($P < 0.05$). Data were analyzed by Student's *t* test.

Adenylate cyclase toxin activity assay. Adenylate cyclase toxin is an important virulence factor for *B. pertussis*, and without it, *B. pertussis* is avirulent (7, 21, 23). The toxin enters phagocytic cells, elevates cyclic AMP (cAMP) levels, and subsequently paralyzes immune defenses such as chemotaxis, phagocytosis, superoxide generation, and microbial killing (1, 6, 13). Because FITC-labeled BP338 seemed to have an altered ability to resist phagocytosis, it was possible that FITC labeling modified adenylate cyclase toxin activity. Therefore, adenylate cyclase toxin activity in bacterial suspensions was measured as [α-^{32}P]ATP converted to [^{32}P]cAMP as previously described (12). BP338 and GFP-expressing BP338 had comparable adenylate cyclase toxin activity, but FITC-labeled BP338 adenylate cyclase toxin activity was reduced fivefold (Table 1), suggesting that FITC modified the adenylate cyclase toxin activity. No activity was seen in BP348 *ptl*::pCW245-2, the adenylate cyclase toxin mutant expressing GFP.

Our studies suggest that FITC labeling compromised at least one extracellular virulence factor, adenylate cyclase toxin. However, we cannot rule out the possibility that other proteins were affected. Such alterations allowed neutrophils to efficiently phagocytose *B. pertussis*, providing misleading results. Clearly, GFP is more appropriate than FITC for labeling *B. pertussis* and studying interactions with human phagocytes. Therefore, care should be taken when using labeled bacteria in phagocytosis assays. Future studies involving GFP-expressing *B. pertussis* are in progress to study the role of human antibodies against *B. pertussis* virulence factors in promoting opsonization and phagocytosis.

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