The EMBO Journal vol.9 no.9 pp.2803 - 2809, 1990

Fimbrial phase variation in *Bordetella pertussis*: a novel mechanism for transcriptional regulation

Rob Willems, Annet Paul, Han G.J.van der Heide, Anja R.ter Avest and Frits R.Mooi

Laboratory of Bacteriology, National Institute of Public Health and Environmental Protection, PO Box 1, 3720 BA Bilthoven, The Netherlands

Communicated by P.H.Mäkelä

Fimbriae belong to a class of extracellular filamentous proteins which are involved in the attachment of bacteria to host tissues. *Bordetella pertussis*, the etiological agent of whooping cough, produces two serologically distinct fimbriae. We show that, like a number of other *B.pertussis* virulence genes, transcription of the fimbrial subunit genes (*fim*) is positively controlled by *trans*-acting polypeptides encoded by the *bvg* locus. In addition to this coordinate control, transcription of the *fim* genes is regulated at an individual level by phase variation. This process is characterized by a switching between a high and low level of expression of a particular *fim* gene. We have identified a conserved DNA region, located close to the start of the *fim* genes, which is likely to be involved in both positive regulation by the *bvg* locus, and phase variation. This promoter region contains a stretch of ~15 C residues and it appears that phase transitions occur by small insertions or deletions in this C-rich region. We propose that these mutations affect transcription of the *fim* genes by varying the distance between the binding site for an activator and the −10 box. The *fim* promoter shows homology with the pertussis toxin promoter, which is also positively regulated by the *bvg* locus.

Key words: Bordetella/fimbriae/phase variation/promoter/regulation

Introduction

Bordetella pertussis is the causative agent of whooping cough or pertussis, a serious respiratory disease. A large number of virulence factors have been implicated in the pathogenesis of pertussis including at least four toxins (pertussis toxin, adenylate cyclase, tracheal cytotoxin and dermonecrotic toxin) and two types of adhesins (the filamentous hemagglutinin, and fimbriae) (see Weiss and Hewlett, 1986 and Mooi, 1988 for reviews). *B.pertussis* produces two serologically distinct fimbriae, designated serotype 2 and serotype 3 fimbriae, which are composed of subunits with slightly different molecular sizes: 22 500 and 22 000 daltons, respectively (Ashworth *et al.*, 1982; Irons *et al.*, 1985; Zhang *et al.*, 1985). We have shown (Mooi *et al.*, 1987) that in addition to the genes coding for the serotype 2 and 3 fimbriae (*fim2* and *fim3*, respectively), *B.pertussis* strains contain a third fimbrial gene (*fimX*) which codes for an as yet unidentified product.

The expression of *B.pertussis* virulence genes is affected by growth conditions such as temperature, and the concentration of $MgSO_4$. Under nonpermissive conditions, i.e. a temperature of 25°C or the presence of 20 mM $MgSO_4$, the genes are repressed. When the cells are subsequently shifted to permissive conditions, production is resumed (Lacey, 1960; Idigbe *et al.*, 1981). Recent evidence indicates that these growth conditions act through the *bvg* locus, which codes for three polypeptides involved in sensory transduction (Stibitz *et al.*, 1988; Knapp and Mekalanos, 1988; Arico *et al.*, 1989). Indeed most *B.pertussis* virulence factors are not produced in strains harbouring transposon insertions in the *bvg* locus (Weiss *et al.*, 1983). Thus most *B.pertussis* virulence genes are part of a single regulon, positively regulated by *bvg*. In addition to this coordinate regulation, fimbrial genes are subject to a second type of control, which occurs independently of other virulence genes. Thus a particular *B.pertussis* strain may produce both types of fimbriae, only one type, or no fimbriae at all. The mechanism underlying this fimbrial phase variation probably involves changes at the DNA level, since the capacity to produce a particular fimbria at a high or low level is a relatively stable characteristic which is passed on to progeny. *In vivo*, presumably under immunological pressure, fimbrial phase variation can be readily observed (Preston *et al.*, 1980). In this paper we identify DNA regions involved in the regulation of transcription of *B.pertussis* fimbrial genes, and discuss the mechanism underlying fimbrial phase variation. Our results suggest that *bvg* regulation and phase variation are linked at the molecular level.

Results

Effect of bvg on expression of fimbrial genes

Two *B.pertussis* strains were used to study the effect of *bvg* on expression of *fim* genes, the Wellcome 28 strain which produces high amounts of serotype 2 and 3 fimbriae, and the Tohama (BP536) strain which produces high amounts of serotype 2 fimbriae, and low amounts of serotype 3 fimbriae (Figure 1). When the Wellcome 28 strain was grown in the presence of 20 mM $MgSO_4$, a condition known to repress *bvg*-controlled genes, no serotype 2 or 3 fimbrial subunits were detected (Figure 1). The presence of $MgSO_4$ also decreased the amount of serotype 2 fimbriae produced by the Tohama strain, although repression was less severe than observed in the Wellcome 28 strain (Figure 1). No fimbrial subunits were detected in a derivative of Tohama (BP347) containing a transposon insertion in the *bvg* locus (Figure 1). These observations indicate that, like a number of other *B.pertussis* virulence genes, the *fim2* and *fim3* genes are subject to *bvg* regulation.

Identification of the fim3 promoter region

Approximately 170 bp upstream of the start codon of *fim3*, several inverted repeats are observed which may function

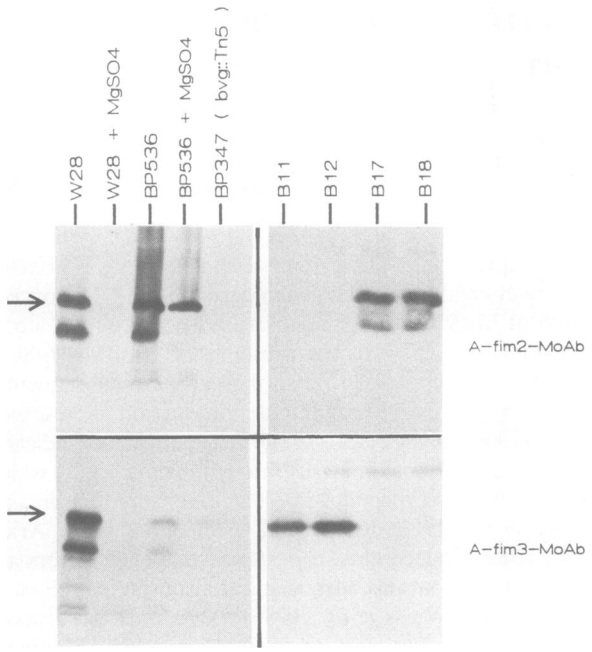

Fig. 1. Production of serotype 2 and 3 fimbrial subunits. Total cell lysates from strains, grown at 37°C in the absence or presence of 20 mM $MgSO_4$, were analyzed by immunoblotting. The blots were incubated with a monoclonal antibody directed against serotype 2 (a-fim2 Moab), or 3 (a-fim3 Moab) subunits. Positions of fimbrial subunits are indicated by arrows. The bands which are sometimes observed below the arrows are proteolytic fragments of the fimbrial subunits. Abbreviations: W28, Wellcome 28.

as (*rho*-independent) transcriptional terminators (Mooi *et al.*, 1989) (Figure 2). Thus it seemed likely that transcription of the *fim3* gene was initiated downstream of these putative terminators, i.e. from within a region located not more than 170 bp from the *fim3* start codon. The following experiment was performed to determine if transcription was indeed initiated from within this region. A 904 bp *Sph*I fragment, derived from the Wellcome 28 strain (Figure 2), was inserted into the multicloning site of pMMB67. The *Sph*I fragment contains one of the putative transcriptional terminators located upstream of *fim3*, and a complete copy of the fim3w$^+$ gene (in naming the *fim* genes we observe the following conventions: 2, 3 or X indicate the serotype; w[ellcome], t[ohama], b11, etc. indicate the strain from which the gene is derived, and + and − indicate whether the gene was expressed at high or low levels in the original strain, respectively). Plasmid pMMB67 contains the *tac* promoter and the *lacI* repressor, and genes cloned into its multicloning site can be expressed at high levels in *E.coli* if the *tac* promoter is derepressed by addition of IPTG (isopropyl-β-thiogalactopyranoside). Two recombinant plasmids which contained the *fim3w*$^+$ gene in the correct or inverse orientation relative to the *tac* promoter, designated pMMB→*fim3w*$^+$ and pMMB*fim3w*$^+$←, respectively, were selected for further study. In *E.coli* strains harbouring pMMB→*fim3w*$^+$, no serotype 3 fimbrial subunits were detected on immunoblots even when the *tac* promoter was derepressed by addition of IPTG (results not shown). This might be due to the fact that transcription initiated from the *tac* promoter is efficiently terminated at the putative *fim3* terminator and/or to proteolysis of the subunit in *E.coli*.

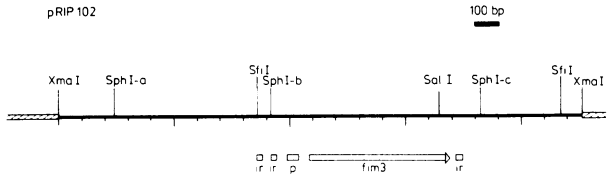

Fig. 2. Physical and genetic map of the DNA region harbouring the *fim3* gene. The thick line and shaded boxes represent *B.pertussis* and pEMBL8 DNA, respectively. Only restriction enzyme recognition sites relevant for this study are shown. The *Sph*Ib−*Sph*Ic DNA fragment was used to detect *fim3* mRNA. Abbreviations: ir, inverted repeat; p, promoter; bp, base pair.

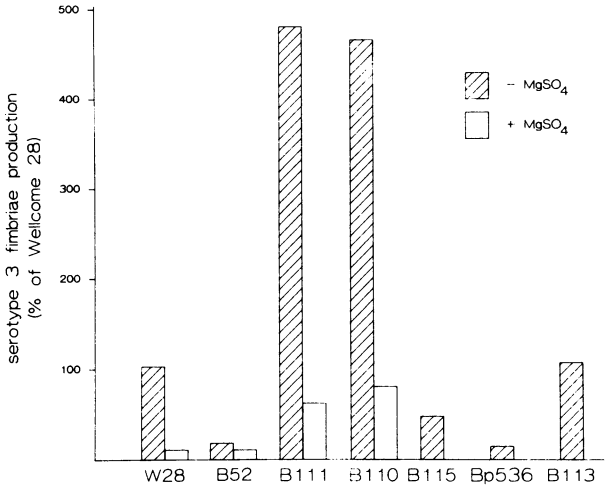

Fig. 3. Production of serotype 3 fimbriae, as determined by a whole cell ELISA. Microtitre plates were coated with cells grown in the presence or absence of 20 mM $MgSO_4$ and the amount of serotype 3 fimbriae was determined with a monoclonal antibody raised against serotype 3 fimbriae. The amount of serotype 3 fimbriae produced by the Wellcome 28 strain was arbitrarily set at 100%. B110, B111 and B115 are derivatives of B52 containing pMMB→*fim3w*$^+$, pMMB*fim3w*$^+$← and pMMB*fim3b18*$^-$←, respectively. B113 was derived from BP536 by passage through a rabbit. Abbreviation: W28, Wellcome 28.

When the plasmids were mobilized into *B.pertussis* strain B52, a derivative of Tohama in which the *fim2* and *fim3* genes have been inactivated by insertions, *fim3w*$^+$ was expressed at high levels (Figure 3). Compared to the Wellcome 28 strain, production was approximately five times higher in B52 carrying pMMB→*fim3w*$^+$. This higher production is probably due to a gene dose effect. Production of fimbriae was independent of the orientation of the *fim3w*$^+$ gene in PMMB67 (Figure 3), indicating that transcription of *fim3w*$^+$ is not dependent on a vector promoter, but initiated from within the *Sph*I fragment. When *B.pertussis* strains harbouring pMMB→*fim3w*$^+$ were grown in the presence of $MgSO_4$, a seven-fold decrease in fimbriae production was observed relative to controls grown without $MgSO_4$ (Figure 3), suggesting that expression of the cloned *fim3w*$^+$ gene was still *bvg*-dependent.

Addition of IPTG did not significantly affect production of fimbriae by *B.pertussis* strains carrying pMMB→*fim3w*$^+$, even when cells were grown in the presence of 20 mM $MgSO_4$ (results not shown), possibly because transcription of *fim3w*$^+$, initiated from the *tac* promoter, is hampered by the putative terminator.

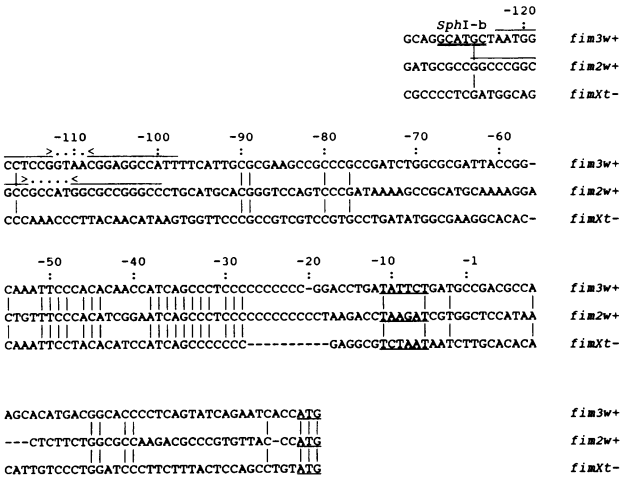

Fig. 4. Comparison of promoter regions of the *fim2*, *fim3* and *fimX* genes. The *fim2* and *fim3* genes are derived from the Wellcome 28 strain, whereas the *fimX* gene is derived from the Tohama strain. The sequences have been aligned by their putative −10 regions and initiation codons (underlined). Bases conserved in all three sequences are indicated by |. Dashes indicate gaps introduced to increase the number of matches. Inverted repeats are overlined. The *Sph*Ib site used to subclone *fim3* genes has been underlined twice. The *fim2* sequence is from Livey *et al.* (1987).

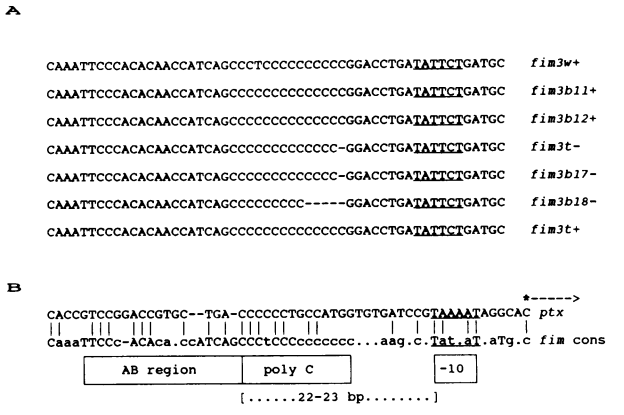

Fig. 5. (**A**) Comparison of promoter regions derived from *fim3* genes in the + or − phases. The sequences have been aligned by their putative −10 regions (underlined). The *fim3t*$^-$ sequence was determined previously (Mooi *et al.* 1989). (**B**) Comparison of the *fim* consensus promoter sequence (cons) with the pertussis toxin (*ptx*) promoter. The putative activator binding region (AB region) and −10 box are indicated in the *fim* sequence. In the *fim* sequence, capital and small letters indicate bases which are conserved in three and two *fim* sequences, respectively. Dots indicate positions where all three *fim* sequences have different bases. Similarities between the *ptx* and the *fim* sequence are indicated by |. Dashes have been introduced to increase the number of matches. The −10 boxes are underlined. The transcriptional start in the *ptx* sequence is indicated by *→ (Nicosia and Rappuoli, 1987).

Comparison of the fim2, fim3 and fimX promoter regions

Because the *fim2*, *fim3* and probably also the *fimX* genes are regulated in a similar way, we reasoned that it should be possible to identify their promoter regions by searching for homology. A comparison of the regions located upstream of the three *fim* genes revealed one area which was well conserved in all three sequences (Figure 4). This area is located 60−90 bases upstream of the initiation codon. It contains an AT-rich sequence, presumably representing a −10 box (Harley and Reynolds, 1987) and further upstream a long stretch of Cs. In the *fimX* sequence, the C stretch is much shorter (~7 residues) than in the *fim2* and *fim3* sequences (15 and 13 Cs, respectively), suggesting that a deletion has occurred in the *fimX* C region.

Interestingly, the *fim2* sequence contains a potentially strong transcription terminator located at approximately the same relative position as in the *fim3* DNA, suggesting a similar transcriptional organization.

DNA sequence analysis of promoter regions derived from fim3 genes in different phases

In addition to the coordinate regulation by *bvg*, the fimbrial genes are controlled individually by a process called phase variation. To determine the mechanism of fimbrial phase variation, we compared the DNA sequence, located between the *Sph*I-b site and the initiation codon of *fim3* genes (Figure 4), derived from strains producing high (Wellcome 28, B11 and B12) or low (Tohama-BP536, B17 and B18) amounts of serotype 3 fimbriae (Figure 1). This region was chosen, because it was shown to be sufficient for high level expression of *fim3* (see above). Cloning of *fim3* alleles was facilitated by the usage of *Sfi*I, a restriction endonuclease which generates unique single-stranded extensions (see Materials and methods). All six sequences analysed were identical except for the regions containing the C stretch (Figure 5A). C stretches preceding *fim3*$^-$ genes were one to five Cs shorter than C stretches preceding *fim3*$^+$ genes. A *Sph*I fragment, harbouring one of the low-expressed *fim3* genes (*fim3b18*$^-$), was cloned into pMMB67 and the resulting plasmid (pMMB*fim3b18*$^-$←) was transferred to strain B52. Compared to strains carrying pMMB*fim3w*$^+$←, the strain with pMMB*fim3b18*$^-$← produced very low amounts of serotype 3 fimbriae (Figure 3). These results indicate that phase transitions between high- and low-expressed *fim3* genes occur by insertions and deletions in the C stretch. They also confirm the assumption that the conserved region located upstream of the *fim* genes represents the *fim* promoter.

Fimbrial phase variation in vivo

We also wanted to compare the *fim3* promoter regions, derived from the same strain, before and after a fimbrial phase switch. Phase switches are observed at a very low frequency *in vitro*. However, *in vivo* they occur readily, presumably due to immunological pressure (Preston *et al.*, 1980). To isolate a derivative of the Tohama strain which had switched on its *fim3* gene, a rabbit was infected with strain BP536 (serotype 2$^+$, 3$^-$) (Figure 1). The strain was reisolated every week, and the serotype of the recovered strain was determined. After ~40 days, a change in serotype was observed as determined by slide agglutination; the strains isolated from the rabbit were of serotype 2$^+$, 3$^+$. One of the isolated strains, designated B113, was analysed by immunoblotting (not shown) and a whole cell ELISA (Figure 3), and it appeared that compared to its parental strain it showed a large increase in serotype 3 fimbriae production. The sequence of the *fim3t*$^+$ promoter region was determined, and it was found that an insertion of a single C had occurred in the C-rich region (Figure 5A), again

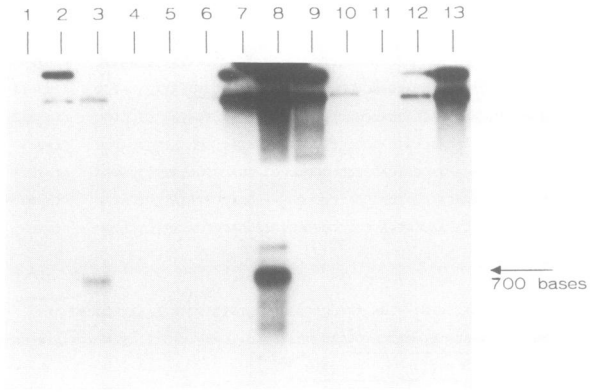

Fig. 6. Northern blot of *fim3*-specific mRNA. RNA (10 μg) was separated on an agarose–formaldehyde gel, transferred to a nylon membrane, and hybridized to a *SphI* DNA fragment derived from *fim3* (see Figure 2). The size of the main *fim3*-specific transcript detected is shown on the right. (**1**) SM10; (**2**) SM10 with pMMB*fim3w*$^+$←; (**3**) Wellcome 28; (**4**) Wellcome 28 grown in the presence of 20 mM $MgSO_4$; (**5**) B52; (**6**) B52 with pMMB67; (**7**) B52 with pMMB*fim3b18*$^-$←; (**8**) B52 with pMMB*fim3w*$^+$←; (**9**) B52 with pMMB*fim3w*$^+$← grown in the presence of 20 mM $MgSO_4$; (**10**) BP347; (**11**) BP347 with pMMB67; (**12**) BP347 with pMMB*fim3w*$^+$←; (**13**) B52 with pMMB*fim3w*$^+$←, treated with RNase.

implicating this region in phase variation, and initiation of transcription.

Detection of fim3-specific mRNA

To confirm that the observed effects of *bvg*, growth conditions and deletions in the C track affected production of fimbrial subunits at the transcriptional level, we characterized *fim3*-specific mRNA by Northern blotting experiments (Figure 6). Differences in copy numbers between plasmids harbouring *fim3* will affect the amount of *fim3* mRNA produced. To determine whether these differences were significant, we looked at the amount of mRNA transcribed from the β-lactamase gene, *bla*, which is located on pMMB67. No large variations were observed in the levels of *bla*-specific mRNA detected in the various strains harbouring pMMB67 and its derivatives (results not shown).

A probe derived from *fim3* was used to detect *fim3*-specific mRNA (Figure 6). The procedure used to isolate RNA generally removed most chromosomal DNA. However, RNA isolated from strains carrying pMMB67 or its derivatives still contained plasmid DNA. Plasmid DNA and RNA could be distinguished by RNase digestion (Figure 6) and by comparing the Northern blots of strains carrying different plasmids, or grown under different conditions.

No *fim3* mRNA was detected in strain B52, presumably because the *fim3* gene in this strain contains an insertion which destabilizes the mRNA. High amounts of *fim3* mRNA were detected in strain B52 with plasmid pMMB*fim3w*$^+$←. The molecular size of the main transcript, 700 bases, corresponds well with the distance between the *fim3* promoter and the strong terminator located downstream of *fim3* (i.e. 700 bp). Northern blots of the Wellcome 28 strain gave essentially the same result, except that the amount of the transcript was smaller. This indicates that the transcriptional organization of *fim3* was not changed due to cloning in pMMB67. The 700 base transcript was not detected when the Wellcome 28 strain, or B52 harbouring pMMB*fim3w*$^+$←, were grown in the presence of $MgSO_4$, or when pMMB*fim3w*$^+$← was transferred to the BP347 strain, which harbours a transposon insertion in the *bvg* locus. These results indicate that this locus is required for transcription of *fim3*. In agreement with this assumption is the observation that no *fim3* mRNA could be detected in *E.coli* strains harbouring pMMB*fim3w*$^+$←. The 700 base *fim3* transcript was also not detected in strain B52 with pMMB*fim3b18*$^-$←, demonstrating that the deletion in the C track, upstream of *fim3b18*, affects transcription of *fim3*. Essentially identical results were obtained with a synthetic 16-mer (see Materials and methods), derived from a central part of *fim3* (results not shown).

Discussion

This study shows that, like a number of other *B.pertussis* virulence factors (Weiss *et al.*, 1983; Arico *et al.*, 1989; Miller *et al.*, 1989; Laoide *et al.*, 1990), production of fimbriae is positively controlled by the *bvg* locus. In addition to this coordinate regulation, the *fim* genes are subject to a second control mechanism, called phase variation, which operates at the level of individual *fim* genes. This phase variation is characterized by a switching between high (*fim*$^+$) and low (*fim*$^-$) level expression of a particular *fim* gene. Our results indicate that a region located 100 bp upstream of the *fim3* gene is involved in both types of regulation. Furthermore, this *fim3* promoter region was found to be conserved in two other *B.pertussis* fimbrial genes, *fim2* and *fimX*. Analysis of *fim3*-specific mRNA revealed a (700 base) transcript, the molecular size of which corresponds with the distance between the *fim3* promoter, and a strong *rho*-independent terminator located downstream of *fim3*. This transcript was not detected in a strain with a transposon insertion in the *bvg* locus, or when strains were grown in the presence of $MgSO_4$. Thus, the *fim3* gene is positively regulated by the *bvg* locus at the transcriptional level.

A characteristic feature of the *fim* promoter is a stretch of Cs, located upstream of the putative −10 box (Figure 4). Analysis of *fim3* genes in the + or − phases revealed that insertions and deletions of 1–5 bp in the C stretch are responsible for fimbrial phase variation. They affect the distance between the putative −10 box and a DNA sequence, tentatively designated AB (for Activator Binding) region, which is well conserved in all three *fim* sequences (Figure 5B). Presumably the distance between the AB region and the −10 box is important for a correct positioning of the RNA polymerase molecule relative to the −10 box. The *fim* promoter resembles the promoter of other positively regulated genes in that it does not contain a standard −35 region (Raibaud and Schwartz, 1984). This type of promoter is probably not recognized efficiently by RNA polymerase in the absence of the activator protein. The distance between the AB region and the −10 box is not identical in the *fim2*$^+$ and *fim3*$^+$ promoter regions, being one base longer in the former (Figure 4). This may suggest some tolerance for additional insertions once a minimal distance of 22 bases has been attained between the two regions. The activator protein, which binds to the AB region, still has to be identified. A possible candidate is a *bvg*-encoded polypeptide. It is also possible that the activator is encoded by a gene which is *bvg*-controlled.

As to the mechanism underlying the mutations in the C stretch, it has been shown that regions with reiterated bases

Table I. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype or genotype	Source or reference
B. pertussis strains:		
Wellcome 28	serotype 2^+, 3^+	Robinson *et al.*, 1985
Tohama derivatives:		
BP536	serotype 2^+, 3^-	Relman *et al.*, 1989
BP347	*bvg*::Tn5	Weiss *et al.*, 1983
B52	serotype 2^-, 3^- (*fim2*::*Sac*I, *fim3*::*kan*)	Mooi *et al.*, in preparation
B110	B52 with pMMB←*fim3w*$^+$	This study
B111	B52 with pMMB*fim3w*$^+$←	This study
B113	serotype 2^+, 3^+, derived from BP536	This study
B115	B52 with pMMB*fim3b18*$^-$←	This study
B162	B347 with pMMB67	This study
B163	B52 with pMMB67	This study
B164	B347 with pMMB*fim3w*$^+$←	This study
Clinical isolates:		
B11	serotype 2^-, 3^+	Mooi *et al.*, 1987
B12	serotype 2^-, 3^+	Mooi *et al.*, 1987
B17	serotype 2^+, 3^-	Mooi *et al.*, 1987
B18	serotype 2^+, 3^-	Mooi *et al.*, 1987
E.coli strains:		
DH5α	*endA1, hsdR17, supE44, thi-1, recAl, gyrA96, relA1, φ80δlacZ M15*	BRL, Maryland, USA
SM10	*thi, thr, leu, supIII*, RP4-2-Tc::Mu, kan	Simon *et al.*, 1983
Plasmids		
pEMBL8	Amp	Dente *et al.*, 1985
pMMB67(HE)	Amp	Furste *et al.*, 1986
pRIP102-T	pEMBL8 with *Xma*I fragment containing Tohama *fim3*$^-$	Mooi *et al.*, 1989
pRIP102-W	pEMBL8 with *Xma*I fragment containing Wellcome *fim3*$^+$	This study
pMMB→fim3w$^+$	pMMB67 with *Sph*I fragment containing Wellcome *fim3*$^+$ in correct orientation relative to *tac* promoter	This study
pMMB*fim3w*$^+$←	pMMB67 with *Sph*I fragment containing Wellcome *fim3*$^+$ in reverse orientation relative to *tac* promoter	This study
pMMB*fim3bl18*$^-$←	pMMB67 with *Sph*I fragment containing B18 *fim3*$^-$ gene in reverse orientation relative to *tac* promoter	This study
pUC19	Amp	Yanisch-Perron *et al.*, 1985

are hotspots for small insertions (duplications) or deletions due to transient misalignment during replication (Streisinger and Owen, 1985).

The *fim* promoter shows some homology with the pertussis toxin promoter (Figure 5B), which is also positively regulated by *bvg* (Nicosia and Rappuoli, 1987). It is significant that the pertussis toxin promoter contains six consecutive Cs at approximately the same relative position as the *fim* promoter. Indeed, when four of the six Cs were deleted, the pertussis toxin promoter was inactivated (Gross and Rappuoli, 1989), suggesting a similar role for the C stretch in the pertussis toxin and *fim* promoters.

In addition to *fim2* and *fim3*, *B.pertussis* harbours at least one other fimbrial gene, designated *fimX* (Mooi *et al.*, 1987; Pedroni *et al.*, 1988). Since no third fimbrial subunit has been described for *B.pertussis*, it seems likely that *fimX* represents a silent gene. This assumption is confirmed by the observation that the distance between the AB region and the −10 box in the *fimX* promoter is 9−10 bases shorter than in promoters derived from highly expressed *fim* genes. For several reasons, activation of the *fimX* gene is a highly unlikely occurrence. The frequency of insertions and deletions in a region with reiterated bases decreases with the length of the repeated DNA (Streisinger and Owen, 1985). Thus this frequency in the C region of *fimX* is probably very low compared to the C regions of *fim2*$^+$ or *fim3*$^+$. Furthermore, an insertion of nine bases is required to optimize the distance between the AB and −10 region. The same holds true for the *fim3b18* gene, isolated from a recent clinical isolate. Apparently, there is no need for *B.pertussis* to express all of its three (or more) *fim* genes.

Phase variation is found in many bacteria and is a means of creating a heterogeneous population from which the environment (for example, the host immune status) can select the best adapted variant. A number of mechanisms have evolved for this type of regulation (Seifert and So, 1988) involving gross DNA rearrangements (translocation or inversion) or small mutations in reiterated sequences. Examples of the latter type of regulation are expression of outer membrane proteins in *Neisseria gonorrhoeae* (Stern and Meyer, 1987), and *Yersinia pseudotuberculosis* (Rosqvist *et al.*, 1988), and expression of lipopolysaccharide in *Haemophilus influenzae* (Weiser *et al.*, 1989). Also, one of the *bvg* genes of *B.pertussis* contains a stretch of six Cs, which has been implicated in switching the phase of this gene (Stibitz *et al.*, 1989). In all the examples involving mutations in reiterated sequences, translation is affected due to the generation of frame shifts. To our knowledge this paper describes the first example of how transient misalignment during replication may also be utilized to regulate gene expression at the level of transcription. Furthermore, it shows how two regulatory phenomena observed in *B.pertussis*, *bvg* control and phase variation, may be linked at the molecular level.

Materials and methods

Strains, plasmids and culture conditions

Strains and plasmids used are listed in Table I. Conditions for growth have been described (Mooi *et al.*, 1987). Antibiotics were used in the following concentrations: kanamycin, 25 μg/ml; ampicillin 100 μg/ml; streptomycin 300 μg/ml.

Bacterial conjugations

Matings were performed as described (Stibitz *et al.*, 1986), except that derivatives of pMMB67 were mobilized into *B.pertussis* with *E.coli* strain SM10. After conjugation, *B.pertussis* exconjugants were selected for on Bordet–Gengou agar plates supplemented with ampicillin and streptomycin.

DNA techniques

Unless stated otherwise, DNA manipulations were performed following the protocols of Maniatis *et al.* (1982).

The *fim3* gene derived from the Tohama strain is located on a 2260 bp *Xma*I fragment which has been completely sequenced (Mooi *et al.*, 1989, and unpublished data). The *fim3* gene from the other *B.pertussis* strains used in this study are located on a similar *Xma*I fragment. The *fim3* gene from the Wellcome 28 strain was cloned by digesting chromosomal DNA with *Xma*I, and separating the DNA fragments by agarose gel electrophoresis. *Xma*I fragments with molecular sizes of ~2300 bp were isolated with porous glass (Geneclean, BIO 101, La Jolla, CA, USA), and inserted into pEMBL8 cleaved with *Xma*I. After transformation, colonies harbouring the Wellcome 28 *fim3* gene were identified by colony blotting, using labelled *fim3* DNA from Tohama as a probe. One recombinant plasmid, designated pRIP102-W, was used for further studies. Later, *fim3* genes were cloned by using *Sfi*I, a restriction endonuclease which generates unique single-stranded extensions because it recognizes an interrupted palindromic sequence. Chromosomal DNA was cleaved with *Sfi*I, and fragments were separated by agarose gel electrophoresis. Subsequently, fragments with a molecular size of ~1300 bp were extracted from the gel as described above, and ligated to the large *Sfi*I fragment of pRIP102-T (Figure 2). This fragment was purified twice by agarose gel electrophoresis to remove the original 1310 bp *Sfi*I fragment derived from the Tohama strain. After cloning the *fim3* genes, the DNA sequence of the region located between the *Sph*Ib site and the initiation codon of *fim3* was determined.

To subclone the *fim3* gene in pMMB67, pRIP102-W was cleaved with *Sph*I, and the 904 bp fragment containing the *fim3* gene was isolated from the agarose gel as described above, and inserted into pMMB67 cleaved with *Sph*I. The orientation of the *B.pertussis* DNA in pMMB67 was determined by digestion with *Sal*I which cleaves asymmetrically in the *Sph*I fragment (Figure 2).

The *fimX* gene was cloned using a synthetic oligonucleotide probe (5'-TTTCGGTCGTCTACCCCTGGA-3') derived from the 3' end of the *fimX* gene (Pedroni *et al.*, 1988). A 5000 bp *Eco*RI–*Pst*I chromosomal DNA fragment from the Tohama strain, which hybridized to the probe, was isolated as described above and cloned into pEMBL8. After transformation, clones harbouring a copy of the *fimX* gene were identified using the probe. The sequence of a DNA region comprising ~200 bp and located upstream of the *fimX* initiation codon was determined.

DNA sequences were determined using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977), combined with pEMBL8, on double-stranded DNA with Sequenase (USB, Cleveland, Ohio, USA) according to the instructions provided by the manufacturer. Both strands were sequenced, and gaps in the sequences were filled in using synthetic oligonucleotides as sequencing primers. Ambiguities in the sequence, due to compressions, were resolved by using 7-deaza GTP in all four sequence reaction mixtures (Mizusawa *et al.*, 1986).

RNA techniques

RNA was isolated as described by Case *et al.* (1988). RNA concentrations were estimated by determining the absorbance at 260 nm. For Northern blot analysis, 2–10 μg RNA was electrophoresed on 1.5% agarose–formaldehyde gels (Selden, 1987), and transferred to a nylon membrane (Hybond-N^+, Amersham, UK). Lambda DNA, digested with *Hin*dIII, was used to calculate the molecular size of transcripts detected. Double-stranded DNA probes were derived from *fim3* (a 904 bp, *Sph*I fragment, see Figure 2), and the β-lactamase gene of pUC19 (a 687 bp *Bgl*–*Ssp*I fragment). DNA was labelled with [α-^{32}P]dCTP by random priming. Hybridizations and washings were performed according to the protocol provided by Amersham for Hybond-N^+.

Fim3-specific mRNA was also identified using a synthetic 16-mer (5'-ATTCCTTCAGCTTGAT-3') derived from a central part of the *fim3* sequence (bases 570–585, Mooi *et al.*, 1989).

To discriminate between RNA and DNA on Northern blots, samples were incubated with 100 μg/ml RNase (Bovine Pancreatic RNase, Boehringer, Mannheim, FRG) for 10 min at 37°C. DNase activity in the RNase preparation was reduced by heating at 100°C for 15 min.

Immunological techniques

Total cell lysates containing ~5 × 10^7 bacteria were analyzed by immunoblotting essentially as described by van Embden *et al.* (1983) using monoclonal antibodies raised against SDS-denatured serotype 2 or 3 fimbrial subunits. The amount of fimbriae produced was determined by a whole cell ELISA. Cells were grown on Bordet–Gengou plates in the presence or absence or 20 mM $MgSO_4$ or 10 mM IPTG (isopropyl-β-thiogalactopyranoside). Subsequently, the cells were scraped from the plates, washed once in PBS (10 mM sodium phosphate, pH 7.4, 155 mM sodium chloride), and suspended to an optical density of 0.002 at 600 nm. 100 μl of the cell suspension was added to microtitre plates, and the water was evaporated overnight at 37°C. The plates were washed with TSTB (1 mM Tris–HCl pH 7.4, 15 mM NaCl, 0.05% Tween 20, and 1% BSA), and blocked by incubation with TSTB for 1 h at 37°C. Fimbriae were quantified by adding monoclonal antibodies directed against the serotype 3 fimbriae to the wells, and subsequently, after washing, a sheep-anti-mouse–peroxidase conjugate. Finally, 100 μl of a peroxidase substrate (0.4 mM 3,3,5,5'-tetramethyl benzidine (Sigma), 0.003% H_2O_2, in 110 mM sodium acetate buffer pH 5.5) was added to the wells, and after stopping the reaction with 100 μl 2 M H_2SO_4, the OD_{450} was determined with a Titertek Multiscan. In some experiments, plates were also coated with a crude preparation of purified fimbriae. The results obtained with these preparations were similar to those obtained with whole cells.

Rabbit experiments

Rabbits were infected as will be described elsewhere (Mooi,F.R., van der Heide,H.G.J., Walvoort,H.C., Brunings,H., Jansen,W.H. and Guinee, P.A.M, submitted.) Briefly, rabbits, weighing 1.5 kg, were infected intranasally with a suspension of 10^6 bacteria. Bacteria were recovered by collecting material from the nasopharynx. This material was directly inoculated onto Bordet—Gengou agar plates, supplemented with the required antibiotics. Colonies were restreaked and serotyped by slide agglutination.

Acknowledgements

We are grateful to Drs Jan D.A.van Embden and Ruurd van der Zee for helpful discussions and critical reading of the manuscript. Monoclonal antibodies were generously provided by Dr Jan Poolman and Betsy Kuipers. Part of this work was supported by a NATO travel grant 0527/87. R.W. and A.P. were supported by the Dutch Organization for Scientific Research (NWO), grant number 900-514-106.

References

Arico,B., Miller,F.M., Roy,C., Stibitz,S., Monack,D., Falkow,S., Gross,R. and Rappuoli,R. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 6671–6675.

Ashworth,L.A.E., Irons,L.I. and Dowsett,A.B. (1982) *Infect. Immun.*, **37**, 1278–1281.

Case,C.C., Roels,S.M., Gonzales,J.E., Simons,E.L. and Simons,R.W. (1988) *Gene*, **72**, 219–236.

Dente,L., Sollazo,M., Baldari,C., Cesareni,G. and Cortese,R. (1985) In Glover,D.M. (ed.), *DNA Cloning–A Practical Approach*. IRL Press, Oxford, Vol. I, pp. 101–107.

Furste,J.P., Pansegrau,W., Frank,R., Blocker,H., Scholz,P., Bagdasarian,M. and Lanka,E. (1986) *Gene*, **48**, 119–131.

Gross,R. and Rappuoli,R. (1989) *J. Bacteriol.*, **171**, 4026–4030.

Harley,C.B. and Reynolds,R.P. (1987) *Nucleic Acids Res.*, **15**, 2343–2359.

Idigbe,E.O., Parton,R. and Wardlaw,A.C. (1981) *J. Med. Microbiol.*, **14**, 409–418.

Irons,L.I., Ashworth,L.A.E. and Robinson,A. (1985) Proceedings of the 4th International Symposium on Pertussis, Geneva, 1984, *Dev. Biol. Stand.*, **61**, 153–163.

Knapp,S. and Mekalanos,J.J. (1988) *J. Bacteriol.*, **170**, 5059–5066.

Lacey,B.W. (1960) *J. Hyg. Camb.*, **58**, 57–93.

Laoide,B.D. and Ullman,A. (1990) *EMBO J.*, **9**, 999–1005.

Livey,I., Duggleby,C.J. and Robinson,A. (1987) *Mol. Microbiol.*, **1**, 203–209.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning–A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Miller,J.F., Roy,C.R. and Falkow,S. (1989) *J. Bacteriol.*, **171**, 6345–6348.

Mizusawa,S., Nishimura,S. and Seela,F. (1986) *Nucleic Acids Res.*, **14**, 1319–1324.
Mooi,F.R. (1988) *Antonie van Leeuwenhoek*, **54**, 465–474.
Mooi,F.R., van der Heide,H.G.J., ter Avest,A., Welinder,K.G., Livey,I., van der Zeijst,B.A.M. and Gaastra,W. (1987) *Microb. Pathogen.*, **2**, 473–484.
Mooi,F.R., ter Avest,A. and van der Heide,H.G.J. (1989) *FEMS Microbiol. Lett.*, **66**, 327–332.
Nicosia,A. and Rappuoli,R. (1987) *J. Bacteriol.*, **169**, 2843–2846.
Pedroni,P., Riboli,B., de Ferra,F., Grandi,G., Toma,S., Arico,B. and Rappuoli,R. (1988) *Mol. Microbiol.*, **2**, 539–543.
Preston,N.W., Timewell,R.M. and Carter,E.J. (1980) *J. Infect.*, **2**, 227–235.
Raibaud,O. and Schwartz,M. (1984) *Annu. Rev. Genet.*, **18**, 173–206.
Relman,D.A., Domenighini,M., Tuomanen,E., Rappuoli,R. and Falkow,S. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 2637–2641.
Robinson,A., Ashworth,L.A.E., Baskerville,A. and Irons,L.I. (1985) Proceedings of the 4th International Symposium on Pertussis, Geneva, 1984, *Dev. Biol. Stand.*, **61**, 165–172.
Rosqvist,R., Skurnik,M. and Wolf-Watz,H. (1988) *Nature*, **334**, 522–525.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Seifert,H.S. and So,M. (1988) *Microb. Rev.*, **52**, 327–336.
Selden,R.F. (1987) In Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D. and Smith,J.A. (eds), *Current Protocols in Molecular Biology*. Greene Publishing Associates, Brooklyn, NY, 4.9.1–4.9.7.
Simon,R., Priefer,U. and Puhler,A. (1983) *Bio/Technol.*, **1**, 784–791.
Stern,A. and Meyer,T.F. (1987) *Mol. Microbiol.*, **1**, 5–12.
Stibitz,S., Black,W. and Falkow,S. (1986) *Gene*, **50**, 133–140.
Stibitz,S., Weiss,A.A. and Falkow,S. (1988) *J. Bacteriol.*, **170**, 2904–2913.
Stibitz,S., Aaronson,W., Monack,D. and Falkow,S. (1989) *Nature*, **338**, 266–269.
Streisinger,G. and Owen,J.E. (1985) *Genetics*, **109**, 633–659.
van Embden,J.D.A., van der Donk,H.J., van Eijk,R.v., van der Heide,H.G.J., de Jong,J.A., van Olderen,M.F., Osterhaus,A.D. and Schouls,L.M. (1983) *Infect. Immun.*, **42**, 187–196.
Weiser,J.N., Love,J.M. and Moxon,E.R. (1989) *Cell*, **59**, 657–665.
Weiss,A.A. and Hewlett,E.L. (1966) *Annu. Rev. Microbiol.*, **40**, 661–686.
Weiss,A.A., Hewlett,E.L., Myers,G.A. and Falkow,F. (1983) *Infect. Immun.*, **42**, 33–41.
Yanisch-Perron,C., Vieira,J. and Messing,J. (1985) *Gene*, **33**, 103–109.
Zhang,J.M., Cowell,J.L., Steven,A.C., Carter,P.H., McGrath,C.C. and Manclark,C.R. (1985) *Infect. Immun.*, **48**, 422–427.

Received on November 11, 1989; revised on June 18, 1990