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Virulence dependent and independent regulation of the *Bordetella pertussis cya* operon

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The *Bordetella pertussis* adenylate cyclase (*cya*) operon is composed of four open reading frames, *cya*A, B, D and E (Glaser *et al.*, 1988, *EMBO J.*, 7, 3997-4004). The *cya*A gene encodes a virulence factor, cyclolysin, a bifunctional protein exhibiting both adenylate cyclase and haemolytic activities while the *cya*B, D and E gene products are necessary for cyclolysin transport. We show that the *cya*A gene is activated by a promoter located 115 bp upstream from the translational start codon and that transcription is only activated in virulent strains. Termination of transcription occurs 3′ to the *cya*A structural gene, however there appears to be some read-through into the downstream genes, resulting in full length *cya*ABDE transcripts. We also identify a second start site of transcription 30 bp upstream from the *cya*B gene, in the intergenic *cya*A-*cya*B region. Transcription is activated from this site in both Vir^+ and Vir^- strains. Thus, the expression of the virulence associated cyclolysin is positively controlled via a *trans*-acting protein encoded by the *bvg* locus while the transport genes show a lower level of constitutive expression which is independent of virulence control.

Key words: adenylate cyclase/haemolysin/regulation of transcription/phenotypic modulation/toxins

Introduction

Bordetella pertussis, the aetiological agent of whooping cough, produces a number of virulence factors including pertussis toxin (Ptx), filamentous haemagglutinin (Fha) and adenylate cyclase toxin (Weiss and Hewlett, 1986). The expression of virulence-associated genes is coordinately regulated by a *trans*-acting protein encoded by the *bvg* locus, originally called *vir* (Weiss *et al.*, 1983; Miller *et al.*, 1989a; Roy *et al.*, 1989). Mutations in the *bvg* locus eliminate expression of virulence factors and result in Vir^- bacteria. A second form of coordinate regulation, known as phenotypic modulation, and brought about by environmental signals, such as $MgSO_4$, nicotinic acid and low temperature (Lacey, 1960), results in the reversible loss of expression of *bvg*-regulated genes.

The molecular mechanism of the regulation of virulence determinants is not completely elucidated. Pertussis toxin and Fha-specific mRNAs are no longer synthesized in Vir^- mutants (Gross and Rappuoli, 1988; Melton and Weiss, 1989; Roy *et al.*, 1989). By creating transcriptional fusions, Miller *et al.*, (1989a) reconstructed a *bvg*-dependent regulatory system in *Escherichia coli* and showed that whereas transcriptional and environmental regulation of *fhaB* is *bvg*-dependent, *ptx* operon expression is not affected by *bvg*. In addition Melton and Weiss (1989) reported that environmental regulation, acting at the level of transcription, might be strain-dependent. It seems, therefore, that the regulatory mechanisms of transcriptional activation of individual virulence determinants could involve additional factors and may also depend on the polycistronic structure of the *bvg*-activated operons.

Adenylate cyclase and haemolysin are major virulence factors produced by *B.pertussis* (Weiss and Hewlett, 1986). Both activities are encoded by a single bifunctional protein, called cyclolysin (Glaser *et al.*, 1988b). The cyclolysin operon is composed of four genes: the structural gene, *cya*A and three genes, *cya*B, D and E, necessary for the secretion of the CyaA protein, and located downstream from the *cya*A gene.

In this paper we analysed the transcriptional organization of the *cya* operon, and its genetic and environmental regulation. We mapped the promoter, upstream from the *cya*A structural gene, and showed that it is virulence-dependent. Most transcripts, initiated at this promoter, terminate within the *cya*A-*cya*B intergenic region. However, there is also a low level of readthrough to produce a full length transcript. In addition, we identified a second weaker promoter located upstream from the *cya*B gene. Finally, we show that this latter promoter is functional in both Vir^- and Vir^+ strains; it is independent of the *bvg* locus and of phenotypic modulating conditions.

Results

Transcription of the cya operon

DNA sequence analysis has provided evidence that the *cya* operon of *B.pertussis* is composed of four open reading frames (ORFs), *cya*ABDE, of ~10 kb in length (Glaser *et al.*, 1988b). The first gene of the operon (*cya*A) encodes a 1706 amino acid (aa) cyclolysin (Glaser *et al.*, 1988a), a bifunctional protein exhibiting adenylate cyclase and haemolytic activities. Secretion of cyclolysin requires the expression of the downstream *cya*BDE genes which have ORFs of 712 aa, 440 aa and 474 aa respectively. The *cya*BDE genes are contiguous while there is a 77 bp intergenic region between *cya*A and *cya*B suggesting that termination of transcription could occur in this region. However, a particular Tn*5* mutant strain, BP349, exhibited adenylate cyclase activity but had a non-haemolytic phenotype (Weiss *et al.*, 1983). Subsequently, the Tn*5* insertion was located to a position two nucleotides downstream from the *cya*A stop codon and was shown to abolish secretion of cyclolysin which suggested that the insertion has a polar effect on the expression of the downstream genes (Glaser *et al.*, 1988b).

To determine the transcriptional organisation of the *cya* operon we characterized *cya*-specific *in vivo* mRNAs by

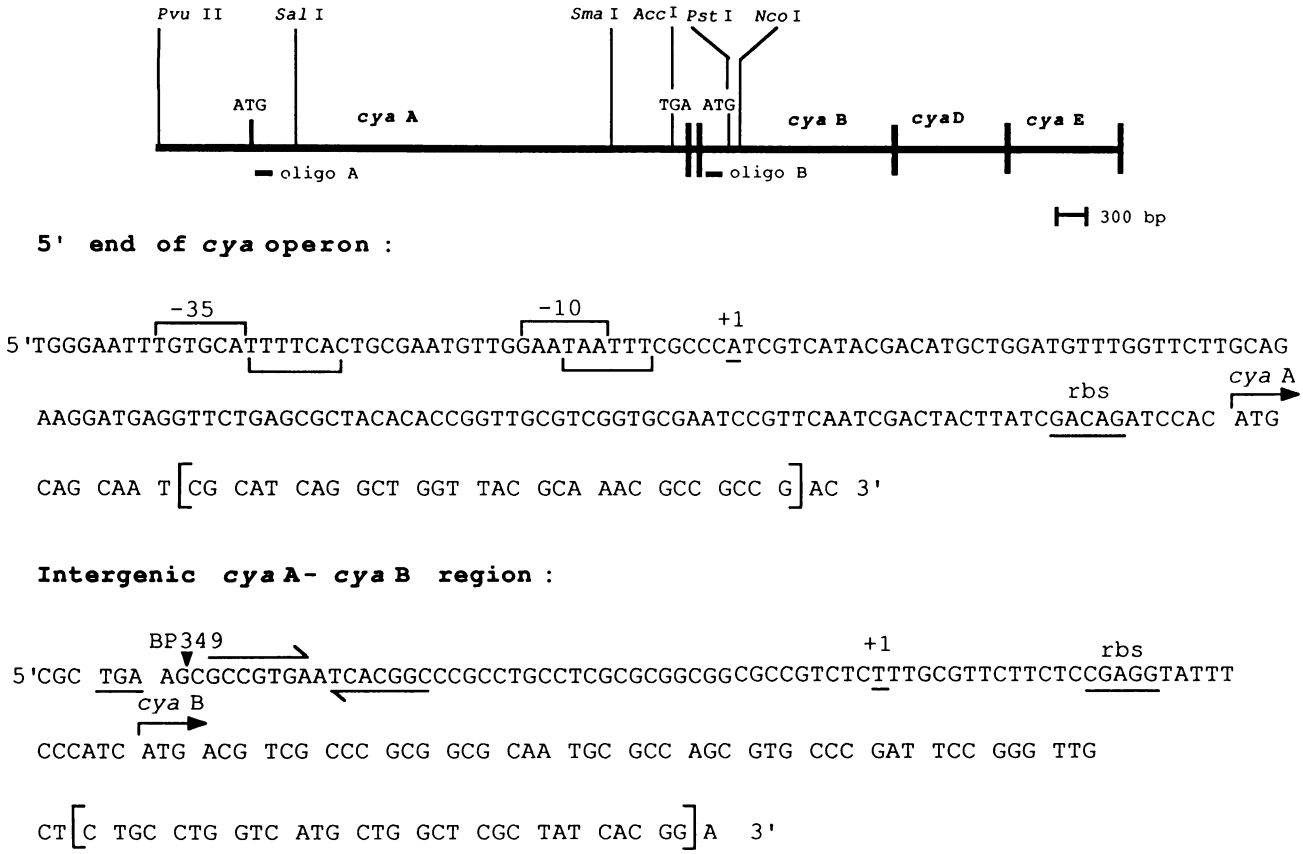

Fig. 1. Top. The *cya* operon, composed of four genes, *cya*A,B,D and E. The 77 bp *cya*A−*cya*B intergenic region is shown by vertical bold lines. The *Pvu*II−*Sal*I fragment, used as a probe for Northern blotting experiments, and the *Acc*I−*Pst*I fragment used for S1 mapping experiments are shown. Oligo A and oligo B refer to the 30 mer oligonucleotides with complementary sequence to the 5′ end of the *cya*A and *cya*B genes respectively, used for primer extension analysis and as probes for Northern blotting experiments. **Bottom.** Sequence of the 5′ end of the *cya*A gene (Glaser *et al.*, 1988a). The start site of transcription (+1) and the two possible promoter sequences are indicated. The putative ribosome binding site (rbs) and the start of the *cya*A structural gene are also shown. A 30 mer oligonucleotide (oligo A) complementary to the sequence shown in brackets was synthesized. Sequence of the intergenic *cya*A−*cya*B region (Glaser *et al.*, 1988b). The TGA *cya*A stop codon is underlined. An inverted repeat sequence, corresponding to a possible rho-independent terminator, is shown with arrows. The start site of transcription (+1), the putative ribosome binding site (rbs) and translational start codon of the *cya*B gene are shown. A 30 mer oligonucleotide (oligo B) complementary to the sequence shown in brackets was synthesized.

Northern blotting experiments. Total RNA from a virulent *B.pertussis* strain was hybridized to *cya*A- or *cya*B-specific probes (Figure 1). A gene probe specific for *cya*A appears to hybridize to a number of RNA species (Figure 2), although the discrete length of these RNAs is not readily apparent. The estimated 4.8 kb transcript probably corresponds to the *cya*A transcript. Higher mol. wt species also hybridize to the probe—these could represent full length 10 kb transcripts— whose half-life might be expected to be very short and therefore difficult to detect. The high background is due to intermediates in the synthesis and degradation of full length transcripts and has been observed by many authors using Northern blots to study different systems (Hagblom *et al.*, 1985; Frank and Iglewski, 1988; Welch and Pellett, 1988; Strathdee and Lo, 1989). RNA from a non-virulent strain did not hybridize to the *cya*A-specific probe.

Using a *cya*B probe, labelled to the same specific activity, a much weaker hybridization signal was detected (Figure 2). This suggests that attenuation occurs 3′ to the *cya*A gene leading to reduced levels of *cya*BDE. Alternatively, the low levels of *cya*B specific transcripts could reflect reduced mRNA synthesis from a second, weaker promoter.

RNA was also prepared from an *E.coli* strain, BNN103, harbouring the *cya* operon on a multicopy plasmid (pDIA5230; gift from P.Glaser). The operon is transcriptionally fused to the *lac* promoter so that *cya* expression is inducible with IPTG and therefore high levels of *cya*-specific messages should be detectable in *E.coli* cells (adenylate cyclase activity is 50- to 100-fold higher than the activity in *B.pertussis* cultures). The data indicate (Figure 3) that the levels of *cya*A-specific transcripts are much higher than the levels of *cya*B specific message and that, at least in *E.coli*, there is readthrough from the strong *lac* promoter into the downstream *cya*BDE genes.

Identification of a virulence-dependent promoter, P_A

The start site of transcription of the *cya*ABDE operon was determined using an oligonucleotide complementary to the 5′ end of the *cya*A mRNA (Figure 1) as a primer for extension analysis. The results show (Figure 4, lane 1) that in a fully virulent *B.pertussis* strain, 18323, transcription starts at an A residue, located 115 bp upstream from the *cya*A translational start codon, ATG (see Figure 1). It is flanked by 5′C and T3′ residues, similar to *E.coli* start sites (Hawley and McClure, 1983; Aoyama and Tukanami,

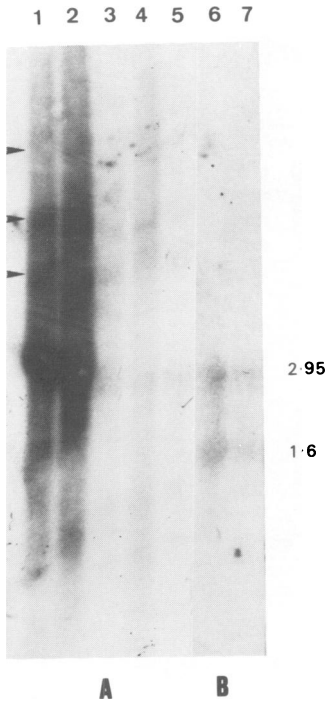

Fig 2. Northern (RNA) blot of *cya*-specific transcripts. Autoradiogram of RNA separated by an 0.9% agarose–formaldehyde gel and hybridized with a *cya*A-specific probe (**A**) or a *cya*B-specific probe (**B**). *B.pertussis* 18323 RNA from a ModX culture (10 μg **lane 1**: 20 μg **lane 2**) and a ModC culture (10 μg **lane 3**; 20 μg **lane 4**) were hybridized to [α^{32}P]dCTP labelled *Pvu*II–*Sal*I *cya*A-specific probe (Figure 1). 20 μg of *E.coli* RNA was used as a negative control (**lane 5**). 20 μg of *B.pertussis* RNA from a virulent strain (**lane 6**) and non-virulent strain (**lane 7**) were hybridized to a [γ^{32}P]ATP labelled oligonucleotide (30 mer) complementary to the 5′ end of *cya*B mRNA (Figure 1) as described in Materials and methods. The estimated sizes of the transcripts shown with arrowheads in lanes 1 and 2 are 10, 6.5 and 4.8 kb. The bands of sizes 2.9 and 1.6 kb are artefactual bands in the region of rRNA migration.

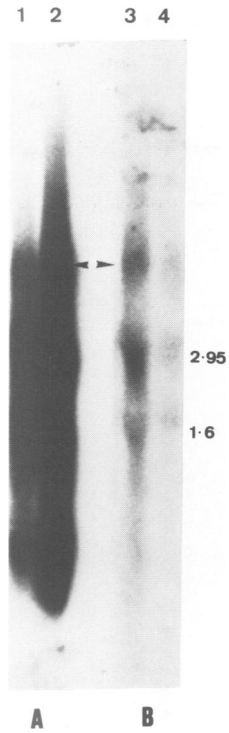

Fig. 3. Northern (RNA) blot of *cya*-specific transcripts in *E.coli*. RNA from an *E.coli* strain BNN103 harbouring the *cya* operon fused to the *lac* promoter (pDIA5203) was hybridized to both *cya*A-specific (**lanes 1** and **2**) and *cya*B-specific (**lanes 3** and **4**) oligonucleotide probes (Figure 1) labelled with [γ^{32}P]ATP. Lanes 1 and 4 contain 5 μg RNA and lanes 2 and 3 contain 20 μg of RNA. The arrowheads identify *cya* transcripts of 5 kb. Artefactual bands (2.95 and 1.6 kb) in the region or rRNA migration are indicated.

1985). This start site is in agreement with that predicted from the nucleotide sequence (Glaser *et al.*, 1988a) and is located 5 or 8 bp downstream from two possible overlapping promoters which show consensus to *E.coli* σ^{70} promoters and to the *B.pertussis ptx* promoter (Nicosia and Rappuoli, 1987).

A reversible transition from a virulent phenotype to a non-virulent phenotype can be achieved by altering bacterial growth conditions (Lacey, 1960). Traditionally this phenotypic modulation is defined as a transition from ModX (Vir^+) to ModC (Vir^-). Strain 18323 was grown under such modulative conditions (in the presence of 5 mM nicotinic acid), total RNA was isolated and was used as a template for primer extension analysis. Figure 4 (lane 2) shows that there is no primer extension product and therefore no transcription initiation upstream from *cya*A in this strain. The same results were obtained using another *B.pertussis* strain (Tohama) grown under non-modulating (ModX, Vir^+; Figure 4, lane 4) and modulating (ModC, Vir^-; Figure 4, lane 5) conditions. As expected, hybridizing total RNA isolated from *E.coli* BNN103 pDIA5230 to the *cya*A-specific probe, transcription was found to initiate at the *E.coli lac* transcription start site and in the presence of IPTG *cya*A message was abundant in *E.coli* cells (Figure 4, lane 6).

A derivative of Tohama strain, BP347, which carries a Tn*5* insertion in the *bvg* operon (Weiss *et al.*, 1983; Arico

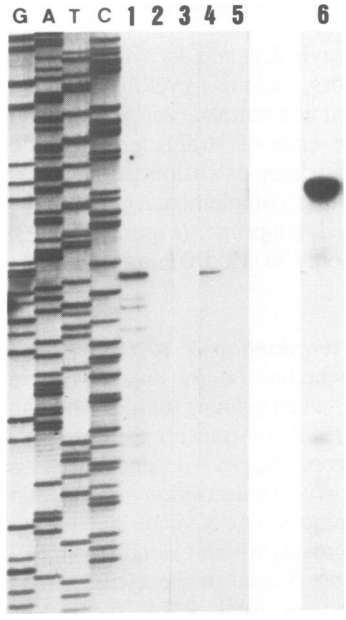

Fig. 4. Primer extension analysis of *B.pertussis* mRNA. Radiolabelled oligonucleotide A (described in Figure 1) was hybridized to total RNA and primer extension was performed with AMV reverse transcriptase. RNAs from Vir^+ (18323 ModX, **lane 1**; Tohama I, ModX, **lane 4**) and Vir^- strains (18323 ModC, **lane 2**; BP347, **lane 3**; Tohama I, ModC, **lane 5**) were used as templates. The same oligonucleotide was used as a primer for dideoxy sequencing, the sequence (of the non-coding strand) is shown. **Lane 6** shows the primer extension product when RNA from IPTG-induced *E.coli* BNN103 pDIA5230 was used as a template.

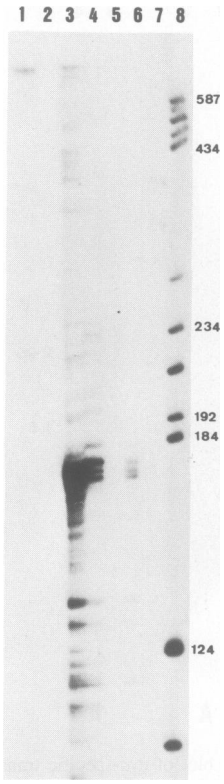

Fig. 5. S1 mapping of the *cya*A transcription termination site. RNAs isolated from *E.coli* BNN103 pDIA5230 (**lane 3**), from *B.pertussis* 18323 ModX (**lane 4**), 18323 ModC (**lane 5**), Tohama I ModX (**lane 6**) and Tohama I ModC (**lane 7**) were hybridized to a 715 bp *Acc*I–*Pst*I DNA probe, labelled at the *Acc*I site (Figure 1). rRNA was used as a negative control (**lane 2**). **Lane 1** shows the undigested denatured probe. **Lane 8** is pBR322/*Hae*III size standard.

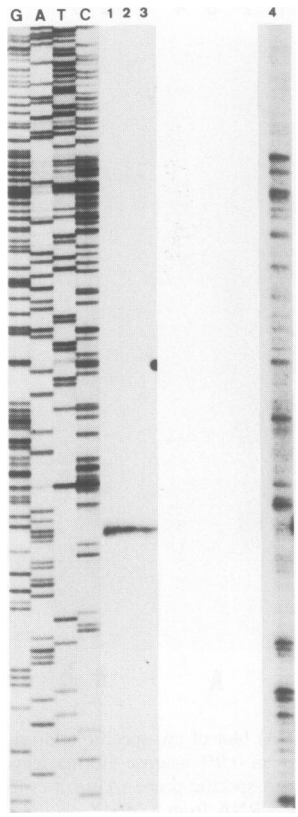

Fig. 6. Primer extension analysis of *B.pertussis* mRNA. Radiolabelled oligonucleotide B (described in Figure 1) was hybridized to total RNA and primer extension was performed with AMV reverse transcriptase. RNAs from Vir^+ (18323 ModX, **lane 1**) and Vir^- strains (18323 ModC, **lane 2**; BP347, **lane 3**) were used as templates. The same oligonucleotide was used as a primer for dideoxy sequencing, the sequence (of the non-coding strand) is shown. **Lane 4** shows the primer extension product when RNA from IPTG-induced *E.coli* BNN103 pDIA5230 was used as a template.

et al., 1989) is avirulent and does not produce any virulence associated factors, including cyclolysin. RNA isolated from BP347 was used as a template for primer extension analysis and, as in the case of strains grown under modulative conditions, no primer extension product was found when an oligonucleotide complementary to the 5′ end of *cya*A mRNA was used as a primer (Figure 4, lane 3). Thus, *cya*A is transcribed only in ModX cultures of bvg^+ *B.pertussis* strains.

Transcription termination 3′ to the cyaA gene

To determine whether the low level of transcripts found in virulent strains, when priming with *cya*B specific sequences, might be the result of transcription termination and low readthrough from the *bvg*-dependent P_A promoter, we looked for *in vivo* transcription termination sites by S1 mapping techniques. A 3′ end labelled probe (715 bp *Acc*I–*Pst*I fragment) which extends from the 3′ end of the *cya*A gene to the 5′ end of the *cya*B gene (see Figure 1) was hybridized to total RNA from Vir^+ and Vir^- *B.pertussis* strains, and also from the *E.coli* strain carrying the plasmid-borne *cya* operon transcriptionally fused to the *lac* promoter. S1 protected fragments of ~170–175 bp were found in the case of virulent *B.pertussis* strains (Figure 5, lanes 4 and 6) and of IPTG-induced *E.coli* strain (Figure 5, lane 3). No S1 resistant hybrids were detected using RNA from non-virulent strains (Figure 5, lanes 5 and 7), in agreement with the results from primer extension analysis.

The protected fragments correspond to a termination site within the *cya*A–*cya*B intergenic region. There is a GC-rich inverted repeat sequence immediately downstream from the TGA translational stop codon which is followed by a sequence rich in thymidines (see Figure 1), suggesting the presence of a rho-independent terminator in this region.

Virulence-independent regulation of the cyaBDE genes

Termination of *cya*A transcripts occur within the 77 bp intergenic region situated between the first gene of the *cya* operon, *cya*A, and the second gene, *cya*B (Figures 1 and 5). We investigated whether there might simply be transcriptional readthrough or whether a second transcriptional start site exists in this region. We used an oligonucleotide complementary to the 5′ end of the *cya*B non-coding strand (Figure 1) as a primer for extension of the *cya*B mRNA. When *B.pertussis* strain, 18323, grown under modulating (Vir^-) and non-modulating (Vir^+) conditions was used a discrete product was observed, corresponding to a transcriptional start site 30 bp upstream from the *cya*B ATG initiation codon (Figure 6, lanes 1 and 2). The same primer extension product was found using RNA isolated from a Vir^- mutant, BP347 (*bvg*::Tn*5*) (Figure 6, lane 3) and also when RNAs from Tohama I ModX and ModC were used as templates (data not shown). Thus, in all the strains tested,

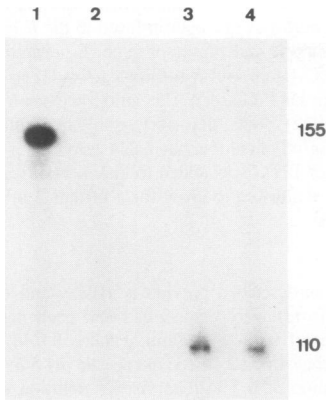

Fig. 7. Comparison of primer extension products using *cya*A-and *cya*B-specific probes. 50 μg of RNA from virulent *B.pertussis* (18323, ModX) was hybridized to radiolabelled oligonucleotide A (**lane 1**) or oligonucleotide B (**lane 3**), primer extension products of 155 and 110 nucleotides respectively, were detected. 50 μg of RNA from non-virulent *B.pertussis* (18323, ModC) was also hybridized to radiolabelled oligonucleotide A (**lane 2**) or oligonucleotide B (**lane 4**), a primer extension product was detected only with oligo B.

transcription initiates at a thymine residue, flanked by 5′C and T3′ residues and contained in a T-rich region, corresponding to the *cya*A transcription terminator region. This start site allows transcription of the *cya*B gene and most likely of the contiguous *cya*D and E genes too. Transcription from this site occurs independently of a functional *bvg* locus and irrespective of modulative growth conditions.

To determine whether this start site is functional in *E.coli*, RNA was isolated from *E.coli* BNN103 pDIA5230. No discrete primer extension product was found using a *cya*B-specific primer (Figure 6, lane 4). This suggests that the transcription initiation site 5′ to the *cya*B gene is not recognized in *E.coli* and that the ladder of primer extension products observed represents readthrough messages from the strong inducible *lac* promoter, in agreement with the Northern blotting data.

To compare the relative promoter strengths of the two promoters, 50 μg of RNA isolated from ModX and ModC cultures of *B.pertussis* was hybridized to either a *cya*A-specific probe or a *cya*B-specific probe labelled to the same specific activity with [^{32}P]ATP and the primer extension reactions carried out under identical conditions (Figure 7). In the case of ModX the level of *cya*A-specific primer extension product is 4- to 5-fold higher (as measured by densitometry), than the level of *cya*B-specific product suggesting that proportionally higher amounts of cyclolysin are synthesized compared to *cya*BDE gene products. However, between ModX and ModC there appears to be no significant difference in the level of *cya*B-specific mRNA (Figure 7, lanes 3 and 4). These results suggest that the second promoter, which activates transcription of the transport genes, *cya*BDE, is much weaker than the P_A promoter and in addition is virulence-independent.

Discussion

The determination of the transcriptional organization of the *cya* operon permits consideration of the relationship between the *cya*A gene and the downstream *cya*BDE genes, between *bvg* locus expression and expression of *cya*, as well as permitting the identification of *B.pertussis* control sequences which are presently poorly understood. It also provides an insight into the regulation of virulence factors and of specific transport genes in gram negative bacteria.

Previous elucidation of the nucleotide and predicted amino acid sequence of the *cya* operon (Glaser *et al.*, 1988a,b) and subsequent comparison with the *E.coli hly* operon (*hly*C, A, B and D; Felmlee *et al.*, 1985) suggested that the organization of both operons is similar in a number of respects. Both systems encode extracellular proteins (cyclolysin and haemolysin) which do not have an N-terminal signal peptide and whose secretion requires the products of the downstream genes (*cya*BDE, *hly*BD). In *E.coli* two haemolysin-specific transcripts have been identified, an abundant *hly*CA species and a less stable *hly*CABD species. The transcripts are activated by a single promoter located 470 nucleotides upstream from the putative *hly*C translational start codon (Welch and Pellett, 1988).

In this work we demonstrate that there are two distinct transcription start sites in the *B.pertussis cya* operon. One initiation site lies 115 bp upstream from the *cya*A structural gene (Figure 1) and is preceded by two possible promoters which show good agreement to the *E.coli* consensus sequence. A similar transcription control region has been found upstream (−45 to −23) from the pertussis toxin (*ptx*) operon of *B.pertussis*, which encodes the five subunits of the Ptx protein (Nicosia and Rappuoli, 1987).

Transcription initiated at the first promoter producing an abundant *cya*A species, terminates 3′ to the *cya*A gene. However, it appears that there is also a low level of readthrough into the downstream genes, resulting in a full length transcript (*cya*ABDE).

A second start site was identified in the intergenic *cya*A−*cya*B region. This was unexpected because genetic analysis using a Tn*5* insertion mutation in this region (Weiss *et al.*, 1983; Glaser *et al.*, 1988b) suggested that the operon was transcribed as one transcriptional unit. The reported leakiness of this mutant strain is probably attributable to low levels of reinitiation from this second transcriptional start site. The Tn*5* insertion site is close to the *cya*B start site (Figure 1) and may have a position effect on the efficiency of transcription from this region. The nucleotide sequence upstream from this site does not show any obvious homology to *E.coli* promoter consensus sequences (Helmann and Chamberlin, 1988). However, evidence is accumulating that the closeness of a sequence to a consensus is insufficient to predict the activity of a relatively weak promoter. It seems likely that alternative σ factors, which have different recognition sequences, will also be found in *B.pertussis* and therefore it is premature to speculate on consensus recognition sequences for *B.pertussis* genes. Alternatively, reinitiation, although specific, may not require additional σ factors due to the proximity of the initiation site to the *cya*A transcriptional termination region.

The level of transcription initiation from the *cya*B start site is considerably lower than the level of transcription initiation from the P_A promoter in virulent strains, probably reflecting the amount of each gene product required by the cell.

In *B.pertussis* a genetic locus, *bvg*A, B and C, originally called *vir*, is required for the expression of many virulence associated factors including Ptx and Fha (Weiss and Falkow, 1984; Stibitz *et al.*, 1988; Gross and Rappuoli, 1988, 1989;

Miller *et al.*, 1989a,b; Roy *et al.*, 1989). The *bvg*-dependent activation of Ptx expression requires a 170 bp sequence upstream from the *ptx* transcription start site. This sequence contains two 21 bp direct repeats and four cytosine residues (Gross and Rappuoli, 1989). In this study we found that the *bvg* locus is also essential for transcriptional activation of the P_A promoter, located 115 nucleotides upstream from the *cya*A gene. We searched for sequences homologous to sequences shown by deletion analysis to be essential for *ptx* expression. No homology was found. Neither is there a run of four cytosines in the *cya* sequence. Roy *et al.* (1989) have recently demonstrated that although both *ptx* and *fha*B require the *bvg* locus for expression, the regulatory mechanisms involved are different. These authors suggest that the *bvg* locus represents the top level of a regulatory hierarchy involved in the control of virulence associated factors. If this is the case then BvgA, in combination with BvgB and BvgC, could directly activate the P_A promoter, or alternatively activation may be indirect, also requiring additional *cya*A-specific factors. These factors could then recognize and bind specific DNA sequences or DNA secondary structures upstream from the *cya*A gene to activate transcription.

It is striking that expression of the contiguous *cya*BDE genes is not dependent on the *bvg* locus. Low level expression of these genes occurs in Vir^- as well as in Vir^+ strains. The *cya*BDE genes encode transport functions required for the secretion of cyclolysin (Glaser *et al.*, 1988b). *cya*B and D are homologous to *hly*B and D, known to be essential for *trans*-membrane transport of *E.coli* haemolysin (Goebel and Hedgepeth, 1982; Mackman and Holland, 1984; Glaser *et al.*, 1988b). HlyB also shows amino acid homology (over ~70% of the sequence) to a membrane glycoprotein, encoded by the *mdr* locus, which confers multidrug resistance phenotype to eukaryotic cells (Gros *et al.*, 1986). Significant homology is also found between the predicted CyaB amino acid sequence and the Mdr protein, suggesting a common ancestor for the corresponding genes.

The efficacy of pathogenic organism, such as *B.pertussis*, in colonizing its host relies on its ability to coordinately express the various virulence factors in a switch-like manner under appropriate environmental conditions. Regulation by the *bvg* locus achieves this coordinate switch efficiently. However, it would also appear to be advantageous to have low level constitutive expression of transport genes, which are involved in the secretion of two major toxic determinants, adenylate cyclase and haemolysin (fused as a bifunctional cyclolysin protein). The presence of low amounts of transport proteins in the cell, under all physiological conditions, would result in the immediate transport of cyclolysin, following activation of the *cya*A gene under favourable conditions. It is interesting to speculate that constitutive expression of the *cya* transport genes has been selected through evolution to permit secretion of other proteins which may not be subject to virulence-associated regulation.

Materials and methods

Bacterial strains, plasmids and culture conditions

The following *B.pertussis* strains were used: 18323 (Pittman, 1984), Tohama I (Institut Pasteur Collection) and a derivative of Tohama I, BP349 (AC^+ Hly^-; Weiss *et al.*, 1983) a kind gift from Dr S.Falkow. For RNA isolation bacteria were grown to exponential phase in Stainer and Scholte medium (Stainer and Scholte, 1971) at 37°C with or without the addition of 5 mM nicotinic acid. The *E.coli* strain used was BNN103 (Δ*lon*) (Young and Davis, 1983). This strain was transformed with plasmids pDIA5230, which carries the *B.pertussis cya* operon fused to the *E.coli lac* promoter, and pDIA17, carrying the *lac*I repressor gene. *B.pertussis cya* expression is inducible by IPTG (isopropyl-β-D-thiogalactoside) in the transformed strain (kind gift from Dr P.Glaser). This strain was grown at 37°C in LB medium (Miller, 1972) containing 100 μg/ml ampicillin (selection for pDIA5230) and 20 μg/ml of chloramphenicol (selection for pDIA17). 1 mM final concentration of IPTG was added to exponentially growing cultures and the bacteria were allowed to grow for a further 2 h before extracting RNA from the cells.

RNA isolation

RNA was isolated as described previously (Blazy and Ullmann, 1990). Culture samples (20 ml) were centrifuged and each pellet was quickly resuspended in 3 ml of 0.02 M sodium acetate, 1 mM EDTA pH 5.3. Sodium dodecyl sulphate in 0.02 M sodium acetate pH 5.3 (3 ml) was added to a final concentration of 0.5%. The viscous solution was immediately transferred to 10 ml of phenol saturated with water and equilibrated at 64°C. The solutions were mixed for 4 min at 64°C and the aqueous phase was re-extracted with phenol once more. The RNA was precipitated by adding sodium acetate to 0.25 M final concentration and 3 vol of ethanol. After overnight incubation at −20°C, the RNA precipitate was collected by centrifugation and dissolved in 1 ml of sterile H_2O. RNA concentration was determined by measuring absorbance at 260 nm and 280 nm. The integrity of the RNA was determined by the presence of discrete rRNA bands after electrophoresis on 1.1% agarose gels.

Northern (RNA) blotting

RNA samples were electrophoresed on horizontal denaturing formaldehyde−agarose gels (Maniatis *et al.*, 1982) and transferred to a nylon membrane (Amersham). Hybridization using a double-stranded 1 kb *Pvu*II−*Sal*I fragment, at the 5′ end of the *cya*A gene (Figure 1), randomly primed with [α^{32}P]dCTP (Amersham) was carried out overnight at 42°C in the presence of 50% formamide. The membrane was then washed four times for 5 min at room temperature with 2× SSC (0.3 M NaCl; 0.03 M sodium acetate pH 7) and 0.1% SDS (sodium dodecyl sulphate). Two higher stringency washes at 50°C for 15 min each were then carried out with 0.1× SSC and 0.1% SDS. When [γ^{32}P]ATP-labelled oligonucleotides were used as probes, hybridizations were carried out at 37°C overnight. The membranes were washed frequently (at least six times, for 5 min each time) at room temperature with 2× SSC and 0.1% SDS, until the levels of radioactivity were significantly reduced. The membranes were then washed once for 15 min at 30°C with 0.2× SSC and 0.1% SDS.

Primer extension analysis

0.07 pmol of [γ^{32}P]ATP-labelled oligonucleotides (Figure 1) was annealed with 50 μg of total RNA by precipitating the DNA and RNA with ethanol, resuspending the pellet in 5 μl H_2O and then heating the mixture to 100°C for 5 min and allowing the temperature to drop slowly to 42°C. 1:10 vol of 10× avian myeloblastosis virus (AMV) buffer (10× buffer: 50 mM Tris−HCl pH 8.3, 500 mM KCl, 100 mM $MgCl_2$, 10 mM DTT), 0.1 mM final concentration αdNTPs and 9 U of AMV reverse transcriptase (Boehringer) were added and the reaction carried out at 42°C for 30 min. 1 μg/ml RNase A was then added and incubation continued for a further 15 min. 7 μl of formaldehyde stop buffer (Amersham) was added and the samples were electrophoresed on 6% polyacrylamide−8M urea denaturing sequencing gels.

S1 mapping

75 μg of total RNA and 25 ng of DNA probe (*Acc*I−*Pst*I fragment labelled with [α^{32}P]dCTP) were precipitated in 3.4 vol ethanol and washed with 85% ethanol. The dried pellets were suspended, by occasional gentle vortexing, in 35 μl of S1 hybridization buffer (80% formamide, 400 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulphonic acid)] pH 6.4, 400 mM NaCl, 1 mM EDTA) and heated to 85°C for 10 min to denature the DNA and the temperature allowed to drop slowly to 60°C overnight to allow hybridization to occur. Nuclease S1 (170 U) was added in 350 μl of S1 digestion buffer (5% glycerol, 0.25 M NaCl, 0.03 M sodium acetate pH 4.5, 1 mM $ZnSO_4$) and the samples were incubated at 37°C for 30 min. The DNA−RNA hybrids were collected by ethanol precipitation, washed with 85% ethanol, dried and then suspended in 10 μl of loading buffer. 5 μl of each sample was electrophoresed on 6% polyacrylamide−8.3 M urea sequencing gel.

DNA sequencing

DNA sequence ladders were prepared using the dideoxynucleotide method of Sanger *et al.* (1977). Oligonucleotides (30 mer) A and B (Figure 1) were used as primers for sequencing the 5′ ends of *cya*A and *cya*B respectively.

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