PN-ABC-887

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: 1/14/88

C7-102

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. C7-102

PR

Attachment

PN-AAC-837
C7-102

Progress Report

For CDR/AID Grant No. DPE-5544-G-SS-7044-00

By Dr. Emanuel Hanski, Department of Hormone Research,

The Weizmann Institute of Science, Rehovot, Israel.

"*Bordetella Pertussis* Invasive Adenylate Cyclase: Its Role in Whooping Cough Pathogenesis and its Possible Use in Development of Acellular Vaccine Against the Disease".

In the second part of the first year of this project we continued to characterize the different forms of *B. pertussis* adenylate cyclase (AC) purified by us recently (see our previous report) and focussed our attempts on the cloning and expression of the *B. pertussis* adenylate cyclase gene.

In collaboration with Drs. Brownlie, Coote and Parton of Glasgow University a 30 kB DNA fragment from the library of wild type *B. pertussis* strain was identified to restore AC and hemolysin activities to *B. pertussis* transposon Tn5-insertion mutant, deficient of both these activities. The Tn5-induced mutant which was avirulent regained its virulence upon restoration of AC and hemolysin activities. The recombinant strain expressed AC in a form which was highly penetrative into target cells. The 30 kB DNA fragment was cleaved into two fragments of 10kB and 3kB. These fragments were cloned into vectors which were under the control of *lac* promotor, and the clones were transformed into *E. coli*. The cloned fragments conferred AC activity to *E. coli*. The AC expressed in *E. coli* had indistinguishable

Rec'd in SCI: OCT 14 1988

biochemical and immunological properties from those expressed in the native bacterium. However, these forms of AC were incapable of penetration into the cell. It is possible, therefore, that a separate component which is expressed in B. pertussis is required for the entry of the catalytic unit of the enzyme into cells. Alternatively, post-translational modification(s) of the enzyme which occur in B. pertussis but not in E. coli produce the invasive form.

The data described above was published recently and the support of your agency is fully acknowledged. Enclosed please find 3 reprints of the paper entitled "Cloning of the adenylate cyclase genetic determinant of Bordetella pertussis and its expression in E. coli and B. pertussis". Microbial Pathogenesis, 1988, 4, 335-344.

C7-102

Rec'd in SCI OCT 14 1988

Microbial Pathogenesis 1988, 4: 335–344

Cloning of the adenylate cyclase genetic determinant of *Bordetella pertussis* and its expression in *Escherichia coli* and *B. pertussis*

Robert M. Brownlie,[1*] John G. Coote,[1] Roger Parton,[1] Joachim E. Schultz,[2†] Arie Rogel[2] and Emanuel Hanski[2]

[1]*Department of Microbiology, Alexander Stone Building, University of Glasgow, Garscube Estate, Bearsden, Glasgow G61 1QH, UK, and* [2]*Department of Hormone Research, The Weizmann Institute of Science, Rehovot, 76100, Israel*

(Received February 9, 1988, accepted February 10, 1988)

Brownlie, R. M. (Department of Microbiology, Alexander Stone Building, University of Glasgow, Gascube Estate, Bearsden, Glasgow G61 1QH, UK), J. G. Coote, R. Parton, J. E. Schultz, A. Rogel and E. Hanski. Cloning of the adenylate cyclase genetic determinant of *B. pertussis* and its expression in *E. coli* and *B. pertussis*. *Microbial Pathogenesis* 1988, 4: 335–344.

A recombinant plasmid, pRMB1, identified from a gene library of *B. pertussis*, restored adenylate cyclase (AC) and haemolysin (HLY) activities to *B. pertussis* BP348 (a Tn5-insertion mutant deficient in both these activities). *B. pertussis* BP348 was considerably less virulent than wild type strains of *B. pertussis* when 3 week-old mice were challenged intranasally; possession of pRMB1 restored virulence. Neither AC nor HLY activities were expressed in *E. coli* harbouring pRMB1. However, expression of calmodulin-responsive AC was obtained in *E. coli* when restriction fragments of pRMB1 were subcloned into other vectors; expression depended on the *lac* and *tac* promoters of these vectors. The enzyme was not readily solubilized from urea extracts of *E. coli* and required sonication for efficient release. One plasmid conferred a specific AC enzymic activity to *E. coli* which was greater than that for wild type *B. pertussis* strains. Unlike extracts of *B. pertussis*, extracts from *E. coli* expressing enzymic AC activity, did not elevate cAMP levels in S49 lymphoma cells. A second plasmid, pRMB2, was identified from the gene library, which contained a *trans*-acting regulatory determinant required for expression of AC, HLY and other virulence-associated factors in *B. pertussis*.

Key words: Adenylate cyclase; *B. pertussis*; cloning; expression; haemolysin; intranasal virulence.

Introduction

Bordetella pertussis produces several factors which have been implicated in the pathogenesis of whooping cough. One of these is an adenylate cyclase (AC) whose properties differ from those of other prokaryotes in being largely extracytoplasmic and activated by the eukaryotic regulatory protein calmodulin. This AC is now recognised as a toxin due to its ability to enter and intoxicate a variety of mammalian cells by elevation of their cAMP levels.[1–5] It has been suggested that AC may assist survival of the bacterium in the host by interfering with phagocyte functions regulated by cAMP; when AC penetrates phagocytes it is activated by calmodulin resulting in a large

* Present address: Department of Microbiology, GBF, Mascheroder Weg 1, D-3300 Braunschweig, West Germany.
† Permanent address: Department of Pharmacy, University of Tübingen, West Germany.

0882-4010/88/050335+10 $03.00/0

© 1988 Academic Press Limited

Table 1 Adenylate cyclase activities in *B. pertussis* strains

Strain	Enzymic activity[a] (nmol cAMP per min per mg protein) Basal activity	With calmodulin	Toxic activity[b] (nmol cAMP per 10^7 cells per mg protein)
165	7.0	2143	8.9
Tohama	4.1	1286	5.1
BP348	0.0	0.3	0.0
BP348 pRMB1	11.6	3529	28.2

[a]Bacteria were harvested from late exponential shake cultures, resuspended in 8 M urea and mixed vigorously. Suspensions were dialysed extensively against 100 mM NaCl, 1 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5, and centrifuged at 160000 × *g* for 60 min. Supernates were assayed for AC activity. Results refer to means of three determinations which differed by less than 6%.

[b]Toxic activity refers to the increase in intracellular cAMP concentration in S49 lymphoma cells after 1 h exposure to the same enzyme extract.

increase in the intracellular cAMP concentration. The importance of AC as a virulence factor was suggested by the finding that a Tn*5* insertion mutant deficient in AC and haemolysin (HLY), strain BP348, was avirulent in suckling mice.[6]

Previously we reported the cloning of the AC genetic determinant of *B. pertussis* on a broad-host-range vector, pLAFR1.[7] The recombinant plasmid, pRMB1 (originally called pLAFR1138), was identified by its ability to restore AC and HLY activities to *B. pertussis* BP348. However, neither of these activities were expressed in *E. coli* harbouring pRMB1. This may have been due to the requirement for a regulatory determinant, *vir*, identified by transposon insertion mutagenesis, required for the expression of AC and other virulence factors in *B. pertussis*.[8] Here we report the subcloning of the AC gene and its expression in *E. coli*, and the cloning of a *trans*-acting regulatory function required for the expression of AC and other virulence determinants in *B. pertussis*.

Results

Adenylate cyclase activities in E. coli *and* B. pertussis *harbouring pRMB1*

AC enzymic and 'toxic' activities were detected in urea extracts of two laboratory strains of *B. pertussis*, Tohama and 165 (Table 1). These activities could only be detected in *B. pertussis* 348 (AC⁻, HLY⁻) when harbouring pRMB1. The AC enzymic and 'toxic' activities of BP348 pRMB1 were 3- and 5-fold higher respectively than those activities obtained for the 'wild type' strain Tohama (the strain from which BP348 was derived). Strain BP348 pRMB1 was also observed to be considerably more haemolytic than strain Tohama. The higher activities conferred by pRMB1 may be due to a gene dosage effect. Neither AC nor HLY activity, however, were detected in *E. coli* CAA8306 (Δ*cya*) harbouring pRMB1. This was presumably due to the absence of the regulatory determinant, *vir*, which is required for expression of virulence factors in *B. pertussis*. *B. pertussis* strain BP347 is a Tn*5*-insertion mutant which does not express several virulence factors because of insertional inactivation of *vir*.[8] Neither AC nor HLY activities were expressed from pRMB1 in strain BP347 (results not shown) confirming the requirement for *vir* for expression of these activities in *B. pertussis*.

Restriction analysis of pRMB1 and hybridization studies

A restriction map of pRMB1 is given in Fig. 1. In Southern blot analysis, a hybridization probe prepared from pRMB1 hybridized to the same *BamH1* fragments from both *B. pertussis* and *B. parapertussis* (strains 10620 and 59321) chromosomal DNA, but not

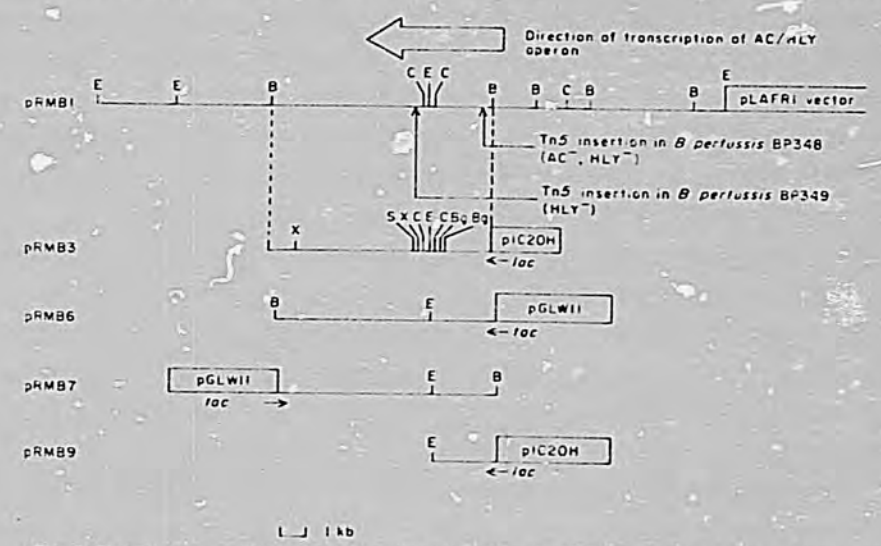

Fig. 1. Restriction maps of plasmids containing cloned *B. pertussis* DNA. Restriction sites are indicated for *BamH1*, B, *Bgl* II, Bg, *EcoR1*, E, *Cla1*, C, *Sac* I, S, *Xho* I, X. The large arrow indicates the proposed direction of transcription of the AC/HLY operon; arrows from *lac* and *tac* indicate the direction of transcription from these promoters.

to any chromosomal fragments from three strains (P-4091, P-4148, P-4480) of the related *B. avium* (results not shown). Tn*5* insertions in BP348 (HLY⁻, AC⁻) and BP349 (HLY⁻, AC⁻) mapped to the same 10 kb *BamH1* fragment (Fig. 1) suggesting that the AC and HLY genetic determinants may be contained in this fragment. Tn*5* insertions are known to cause polar mutations and the positions of the Tn*5* insertions in strains BP348 and BP349 suggest the direction of transcription indicated in Figure 1.

Intranasal infection of mice with B. pertussis

The virulence of strain BP348 and BP348 harbouring pRMB1 was assessed by intranasal infection of 3-week-old mice. Strain BP348 was considerably less virulent than the 'wild type' strains Tohama and TAB1 (the strain from which the genomic library was prepared) when disease was monitored by mouse weight gain, lung pathology and leukocytosis (Table 2). However, when strain BP348 harboured pRMB1, virulence was restored. In several experiments, BP348 pRMB1 was found to have greater lethality than the 'wild type' strains. *In vitro*, pRMB1 was maintained in only 70% of colonies of BP348 after one subculture in the absence of tetracycline. Yet abundant HLY⁻ tetracycline resistant colonies were isolated from the lungs of mice 3 weeks after infection suggesting that there was selection *in vivo* to maintain the plasmid. Although strain BP348 appeared to have reduced virulence in terms of the parameters used, infection persisted since non-haemolytic colonies could still be recovered from the lungs (Table 2). Significant leukocytosis, which is a manifestation of pertussis toxin released in the lungs, occurred in mice infected with all strains other than BP348. Taken together these results suggest that a lower level of infection occurred with strain BP348.

Table 2 Intranasal virulence of *B. pertussis* strains for mice

Strain	Challenge dose (cfu per mouse)	Indicators of infection 21 days after challenge: Deaths[a]	Loss of weight gain[b]	Lung pathology[c]	Recovery from lungs (and phenotype)[d]	Leukocytosis[e]
Tohama	9.4×10^6	0	++	...	...	...
	9.4×10^5	1	+	2.3	+++ (HLY⁻)	...
	9.4×10^4	0	−	1.8	++ (HLY⁻)	HS
TAB1	10.7×10^6	2	+	...	...	...
	10.7×10^5	1	+	3.0	++ (HLY⁻)	...
	10.7×10^4	0	−	1.6	+ (HLY⁻)	HS
BP348	18.0×10^6	0	−	...	...	...
	18.0×10^5	0	−	1.5	++ (HLY⁻)	...
	18.0×10^4	0	−	0.8	+ (HLY⁻)	NS
BP348 pRMB1	7.5×10^6	6	++	...	...	...
	7.5×10^5	6	++	3.3	+++ (HLY⁻)	...
	7.5×10^4	0	−	2.0	+ (HLY⁻)	HS

[a] Number of deaths out of ten mice challenged.
[b] Average mouse weight gain was < 50% (++), < 75% (+) or > 75% (−) of that of uninfected control mice.
[c] The gross lung pathology was assessed and given a score from 0–4 (no abnormalities to gross pathological changes in all lobes). Results refer to average scores of surviving mice.
[d] To determine lung infection, portions of lung were smeared onto BG plates or BG plates with tetracycline (10 μg/ml) and incubated at 37°C for 4–5 days. The extent of growth was assessed as 0 to +++ (no growth to confluency). HLY⁻ refers to haemolytic colonies and HLY⁻ refers to non-haemolytic colonies.
[e] The $\log_{10}$ leukocyte counts from surviving mice were compared with uninfected control mice using a one-tailed Student *t* test; HS = highly significant ($P < 0.01$), NS = not significant ($P > 0.05$).
[f] ... = not tested.

Complementation of B. pertussis *strains BP347 and L84 phase IV*

Although AC activity was not expressed in BP347 harbouring pRMB1, another plasmid, pRMB2, was identified which restored HLY activity to this strain. Further analysis showed that this plasmid also restored AC and other virulence associated factors to strain BP347 (Table 3). It is presumed that pRMB2 contained the *vir* regulatory function which, acting in *trans*, allowed the expression of the chromosomally-encoded virulence determinants. This plasmid also restored expression of several virulence factors to a degraded or so-called phase IV strain, L84IV. A characteristic feature of phase IV strains is their ability to grow on nutrient agar, unlike the phase I strains from which they are derived.[10] Strain L84IV harbouring pRMB2 still retained the capacity to grow on nutrient agar.

Table 3 Complementation of *B. pertussis* strains BP347 and L84 phase IV

Strain	Phenotype[a]: AC	HLY	PT	FHA	HLT	Congo red uptake[b]	Growth on nutrient agar
BP347	−	−	−	−	−	−	−
BP347 pRMB2	+	+	−	+	+	+	−
L84IV	−	−	−	−	−	−	+
L84IV pRMB2	+	+	−	+	+	+	+

[a] PT = pertussis toxin, FHA = filamentous haemagglutinin, HLT = heat labile toxin.
[b] The ability of colonies to take up Congo red from the growth medium has recently been shown to be absent in strain BP347 and phase IV strains.[9]

Table 4 Growth of *E. coli*, containing cloned *B. pertussis* DNA, on various carbon sources[a]

Strain	Sole carbon source: Glucose	Lactose	Maltose	Arabinose
DH1	+++++	+++++	+++++	+++++
CAA8306 (Δcya) pIC2OH	+++++	−	−	−
CAA8306 pRMB1	+++++	−	...[b]	...
CAA8306 pRMB3	+++++	+++	++	+

[a] Strains were grown on M9 minimal agar plates with the listed sugars included as sole carbon sources (0.4% w/v final conc.). Plus signs indicate degrees of growth from complete growth (+++++) to poor growth (+) or no growth (−).
[b] ... = not tested.

Expression of B. pertussis *AC in* E. coli

The 10 kb *BamH1* fragment of pRMB1 was subcloned into the vector pIC2OH[11] to give the plasmid pRMB3 (Fig. 1). Initially it was anticipated that expression of AC may have required the presence of the *B. pertussis vir* locus. Therefore both pRMB2 (containing *vir*) and pRMB3 were transferred by transformation into the same *E. coli* host. As a convenient means to detect expression of AC, plasmids were transferred to a Δ*cya* strain of *E. coli*, CAA8306; production of AC was noted by blue growth on X-GAL plates indicating expression of β-galactosidase which in turn is dependent on cAMP. Unexpectedly, *E. coli* harbouring pRMB3 alone, as well as *E. coli* harbouring both pRMB2 and pRMB3, grew as blue colonies; whereas *E. coli* harbouring either pIC2OH or pRMB2 alone grew as white colonies. In addition, pRMB3 enabled strain CAA8306 to grow on minimal medium containing lactose, maltose or arabinose as sole carbon sources (Table 4). However, growth was not as abundant as for a Cya⁺ strain of *E. coli* (strain DH1).

The orientation of the *BamH1* fragment in pRMB3 suggested that the AC gene might be expressed from the *lac* promoter contained in pIC2OH (Fig. 1). DNA cloned into the *BamH1* site of pIC2OH may result in a fusion protein containing the six amino-terminal amino acids from β-galactosidase together with five amino acids encoded by the polylinker. The 10 kb *BamH1* fragment was subcloned into a second expression vector, pGLW11 (obtained from Dr I. Hunter, University of Glasgow) in two orientations to give plasmids designated pRMB6 and pRMB7 (Fig. 1). DNA cloned in the *BamH1* site of pGLW11 may be expressed from a *tac* promoter and β-galactosidase ribosome-binding site contained in the vector; expression, however, requires a translational start codon from the insert and hence no fusion proteins are produced. *E. coli* CAA8306 harbouring pRMB6 grew as blue colonies whereas the same strain harbouring pRMB7 grew as white colonies on X-GAL plates. This indicated that expression of AC depended on the orientation of the *BamH1* fragment and that the insert contained its own start codon.

The 3 kb *BamH1/EcoR1* fragment, to which the Tn*5* insertion of strain BP348 mapped, was also subcloned into pIC2OH to give pRMB9 (Fig. 1); pRMB9 also allowed expression of β-galactosidase in strain CAA8306. None of the above plasmids bestowed HLY activity to *E. coli* when grown on BG plates.

Extraction of AC enzymic activity from E. coli

Initial experiments indicated that AC enzymic activity was considerably lower in *E. coli* harbouring either pRMB3, pRMB6, or pRMB9 than for wild type *B. pertussis*.

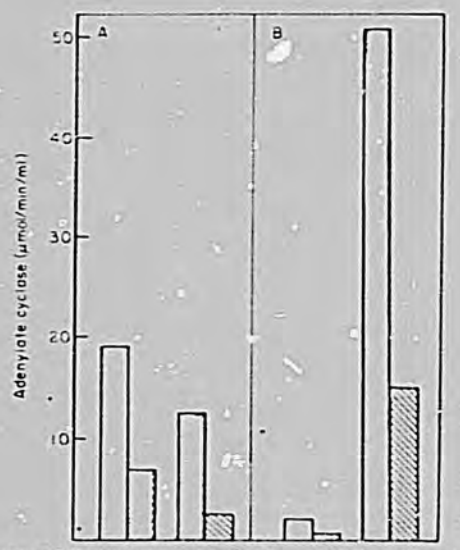

Fig. 2. Efficiency of extraction of *B. pertussis* adenylate cyclase from *B. pertussis* (strain 165) (A) and *E. coli* pRMB9 (B). Bacteria were harvested from late exponential cultures and 2.6 g (wet weight) was resuspended in 16 ml 8 M urea. After vigorous mixing, samples were diluted 200-fold in phosphate buffered saline and assayed for AC activity in the presence of calmodulin (open bars). The suspension was extensively dialysed against 10 Tris-HCl pH 7.5, 100 mM NaCl, 1 mM $CaCl_2$. After centrifugation at 160 000 × *g* for 60 min, AC activity was determined in the supernate (circled bars). The pellet was resuspended in 16 ml 8 M urea and sonicated three times for 30 s using a Branson sonifier at maximal output. AC activity was determined from sonicated suspension after dilution as above (shaded bars). The suspension was centrifuged as above and AC activity was determined from the supernate (hatched bars).

Later experiments, however, showed that more rigorous conditions were required to solubilize substantial quantities of the enzyme from *E. coli* cell extracts (Fig. 2). Only when the particulate material of urea extracts of *E. coli* was sonicated was a substantial level of AC activity released.

AC enzymic activity was detected in sonicated extracts of *E. coli* harbouring either pRMB3 or pRMB9 but not the vector, pIC2OH (Table 5). Activity was increased 250-fold when calmodulin was present. *E. coli* pRMB9 gave the higher activity (perhaps

Table 5 Adenylate cyclase activity in *E. coli* harbouring cloned *B. pertussis* DNA

Strain	Enzymic activity* (nmol cAMP per min per mg protein)	
	Basal activity	With calmodulin
CAA8306 (Δcya) pIC2OH	0.0	0.0
CAA8306 pRMB3	3.3	823
CAA8306 pRMB9	11.3	2950

*Bacteria were harvested from late exponential shake cultures, resuspended in 8 M urea, sonicated thrice for 30 s, and dialysed extensively in 100 mM NaCl, 1 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5. After centrifugation at 160 000 × *g* for 60 min, AC activity was determined in the supernates. Results refer to means of three determinations.

due to the smaller size of insert allowing a higher copy number) and this was higher than for 'wild type' strains of *B. pertussis* (Table 1). None of the *E. coli* extracts were able to elevate cAMP levels in S49 lymphoma cells.

Discussion

A recombinant plasmid, pRMB1, was identified which restored AC and HLY activities to the Tn*5* insertion mutant, *B. pertussis* BP348 (AC^-, HLY^-). The ability of pRMB1 to complement in *trans* would suggest that the plasmid contained the promoter of the operon as well as the AC and HLY genetic determinants. AC enzymic activity was several-fold higher in strain BP348 pRMB1 than in *B. pertussis* Tohama and this was paralleled by an increase in AC toxic activity. It is not clear how the AC is able to penetrate mammalian cells. It has been suggested that the AC may have an A/B structure typical of other bacterial toxins;[3] the A moiety being required for enzymic activity and the B moiety being required for the binding and penetration required for toxic activity. The parallel increase in toxic activity with enzymic activity would suggest that pRMB1 encodes for the entire toxin. It was also observed that strain BP348 pRMB1 gave a larger zone of haemolysis on BG plates than wild type strains. The larger quantities of AC and HLY produced by *B. pertussis* harbouring pRMB1 may be helpful in purification studies.

In Southern blot hybridization studies, a probe prepared to pRMB1 hybridized to the same *BamH1* fragments of *B. pertussis* and *B. parapertussis* DNA but not to any fragments of *B. avium* DNA. This is not totally unexpected as *B. pertussis* and *B. parapertussis* are closely related and both produce active AC. On the other hand *B. avium* is not so closely related to either of these strains and does not produce active AC;[12] the above observation indicates that this is not merely because of mutations, but due to fundamental genetic differences.

Weiss *et al.*[6] previously reported strain BP348 to be avirulent in 7-day-old mice infected intranasally ($LD_{50} > 3\times10^7$ cfu; cf. wild type strain, $DL_{50} = 2\times10^3$ cfu). This animal model is essentially a lethal pneumonia. In the present study strain BP348 was also found to be considerably less virulent than 'wild type' strains of *B. pertussis*. Here, 3-week-old mice were infected intranasally and virulence was assessed using a number of parameters. The plasmid pRMB1 restored virulence to strain BP348 and increased lethality when compared with the parent strain Tohama. These findings add further weight to the importance of AC as a virulence factor in the mouse intranasal infection. The observation that pRMB1 was only 70% stable *in vitro* and yet abundant HLY^+ colonies were isolated from mice 21 days after infection with strain BP348 pRMB1 suggests preferential survival in the mouse for *B. pertussis* expressing AC and HLY. Although strain BP348 had reduced virulence with respect to several indicators of disease such as leukocytosis, lethality, and mouse weight gain, the infection persisted for at least 21 days. This is consistent with the findings of Weiss *et al.*[6] who also found strain BP348 to persist in suckling mice after intranasal challenge.

The role of HLY in infection is not certain but Weiss *et al.*[6] previously found strain BP349 (HLY^-, AC^+) to have an LD_{50} 200-fold greater than the wild type strain; however, strain BP349 had only 20% of AC activity compared to the parent strain.

Thus while AC and possibly HLY may be important virulence determinants required for full manifestation of the disease in the mouse, they may not be essential for colonization and persistent infection. Perhaps *in vivo* release of pertussis toxin enables low level persistent infection of strain BP348. Pittman *et al.*[13] found that 3-week-old mice intranasally infected with a sublethal dose of *B. pertussis* showed several parallels with human infant pertussis such as duration of infection, leukocytosis and possibly

hypoglycaemia. However, the relevance of AC and HLY to human whooping cough remains to be established.

Weiss *et al.*[8] reported that strain BP347 was deficient in AC, HLY, pertussis toxin, heat-labile toxin and filamentous haemagglutinin due to a single Tn*5* insertion in the chromosome. It was proposed that Tn*5* insertional inactivation of a *trans*-acting regulatory function, *vir*, prevented the expression of several virulence determinants in strain BP347. In the present study a recombinant plasmid, pRMB2, was identified which complemented strain BP347 and restored expression of the above virulence factors to this strain. This confirmed the ability of *vir* to act in *trans*. It has long been known that continual subculture of *B. pertussis* may result in degradation of virulent (or phase I) strains to avirulent (or phase IV) strains deficient in several virulence factors.[10] An additional feature of phase IV strains is their tolerance to substances in conventional media which inhibit the growth of phase I strains. However, it is unclear as to whether phase variation (as the change is known) is a step-wise process or due to a single genetic event. Here we have shown that pRMB2 restored expression of several virulence factors to the phase IV strain L84IV. This indicated that the structural genes of these virulence factors are all functional in this strain and that the loss of their activity was presumably due to mutational events in the *vir* locus. Strain L84IV harbouring pRMB2 still retained the ability to grow on nutrient agar indicating that this phenotype is unrelated to *vir* activity. Continual subculture of *B. pertussis in vitro* perhaps selects for two phenotypes independently; firstly, Vir strains freed from the metabolic burden of synthesizing virulence factors which are no longer required; and secondly strains which, through mutations causing alterations in the cell surface, become more resistant to inhibitors in conventional medium.

Although AC or HLY activity were not expressed in *E. coli* harbouring pRMB1, presumably because of the requirement for *vir* for expression of these activities in *B. pertussis*, it was possible to get expression of AC enzymic activity in *E. coli* by use of *lac* and *tac* promoters. As with *B. pertussis*, AC activity in *E. coli* was markedly enhanced (250-fold) by the presence of calmodulin. The inability of the AC expressed in *E. coli* to elevate cAMP levels in mammalian cells may be due to several reasons: a second factor required for penetration of mammalian cells by AC, or functions required for processing of the enzyme may not be encoded by either pRMB3 or pRMB9; alternatively, degradative enzymes in *E. coli* may cause loss of toxic activity.

The reason why none of the plasmids bestowed HLY activity to *E. coli* is not known but little is known of the factors required for the HLY activity of *B. pertussis*.

Recently Glaser *et al.*[14] reported the cloning and expression of the *B. pertussis* AC in *E. coli*. A DNA fragment was sequenced and an open reading frame (ORF) encoding for a polypeptide of 1706 amino acids was identified. A comparison of restriction maps suggests that pRMB3 contains the entire ORF, whereas pRMB9 contains only the 5′ end of the ORF (2.6 kb) which encodes for the calmodulin-responsive catalytic domain of the polypeptide. Glaser *et al.*[14] reported a much lower level of expression of AC enzymic activity in *E. coli* than reported here and this may reflect differences in the vectors and methods of extraction used.

Brezin *et al.*[15] reported that antibody to AC purified from the culture supernate of *B. pertussis* passively protected suckling mice from an otherwise lethal intranasal challenge. In view of this and the relative avirulence of strain BP348 in mice, detoxified AC may be worthy of consideration for inclusion in future acellular vaccines. The high level of 'non-toxic' AC expressed from pRMB9 may prove useful for this purpose. Further work is required to determine if this has protective activity in animal models.

Materials and methods

Bacterial strains and culture conditions. *B. pertussis* strains Tohama and 165 were previously described by Sato and Arai[16] and Hanski and Farfel[2] respectively. *B. pertussis* L84 phase IV and *B. parapertussis* strains were obtained from the National Collection of Type Cultures. *B. avium* strains were obtained from R. B. Rimler.[17] All other bacteria were described previously.[7] Except where stated *E. coli* strains were grown on nutrient agar (Oxoid) plates or with shaking in nutrient broth (Oxoid). X-GAL plates consisted of nutrient agar with 50 μg/ml X-GAL (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) and 0.5 mM IPTG (β-D-thiogalactopyranoside). M9 medium was as described by Maniatis *et al.*[18] *B. pertussis* was grown on Bordet-Gengou medium (BG) and in Stainer and Scholte liquid medium as described previously.[7]

DNA techniques. The gene library of *B. pertussis* was constructed from chromosomal DNA of strain TAB1 as described previously.[7] Restriction mapping, Southern blotting (using high stringency conditions) and ligations were done as described by Maniatis *et al.*[18] Conjugations were done as described previously[7] and transformation was done according to the procedure of Dagert and Ehrlich.[19]

Mouse intranasal infection. *B. pertussis* grown for 24 h on BG plates was resuspended in casamino acids solution (10 g/l casamino acids (Difco technical), 5 g/l NaCl, 0.1 g/l $MgCl_2$, $6H_2O$, 0.016 g/l $CaCl_2$, pH 7.1). The opacity of the suspension was standardized against the WHO 5th International Reference Preparation of Opacity[20] and diluted in 10-fold steps to provide challenge dilutions and for the viable counts. Three-week-old mice of the HaM/ICR strain (Charles River UK Ltd, Manston Road, Margate, Kent CT9 4LT) were infected in groups of ten. After ether anaesthesia, 0.05 ml of challenge dilution was deposited on the external nares and allowed to be inhaled. Weights and deaths were recorded daily. After 21 days of infection, various indicators of infection in surviving mice were monitored.[13] Leukocyte counts of blood collected in heparinized capillaries from the retroorbital plexus of ether anaesthetized mice were made using a Coulter counter (Model Fn, Coulter Electronics, Dunstable, Bedfordshire, UK).

Adenylate cyclase assay. Adenylate cyclase activity was determined by the method of Salomon *et al.*[21] The assay was performed in a final volume of 50 μl at 37°C containing 1 mM [α-^{32}P] ATP (10–40 dpm/pmol), 10 mM $MgCl_2$, 0.1 mM $CaCl_2$ and other ingredients as outlined earlier.[2] When stated, bovine brain calmodulin was present in the assay mixture at a final concentration of 2 μg/ml.

Determination of cAMP levels in S49 murine lymphoma cells. S49 lymphoma cells were grown and washed as described previously.[7] Cells ($1–2 \times 10^6$) were mixed with dialysed bacterial extracts in a final volume of 0.5 ml for 1 h at 37°C. Cells were then pelleted and lysed with 0.5 ml 50 mM sodium acetate, pH 4.2. The cAMP concentration was determined using the method of Gilman.[22]

Protein. Protein was determined by the methods of Lowry[23] and Bradford.[24]

We are grateful to Dr A. Weiss for *B. pertussis* strains BP347 and BP348, Dr R. Rimler for *B. avium* strains, Dr I. Hunter for the vector pGWL11 and to Mr D. MacGregor for *B. avium* DNA. This work was in part funded by the Medical Research Council, UK, and equipment was made available by the Wood Boyd fund of the University of Glasgow Medical Research Fund. Work in Israel was supported in part by grants from the U.S.–Israel Co-operative Development Research Program and the U.S.–Israel Binational Science Foundation.

References

1. Confer DL, Eaton JW. Phagocyte impotence caused by an invasive bacterial adenylate cyclase into mammalian cells. Science 1982; 217: 984–50.
2. Hanski E, Farfel Z. *Bordetella pertussis* invasive adenylate cyclase. J Biol Chem 1985; 290: 5526–32.
3. Hewlett EL, Weiss AA, Crane JK, Pearson RD, Anderson HJ, Myers GA, Evans WS, Hantske LL, Kay HD, Cronin MJ. *Bordetella* extracytoplasmic adenylate cyclase: actions as a bacterial toxin. Dev Biol Stand 1985; 61: 21–6.

C7-102

Progress Report

For CDR/AID Grant No. DPE-5544-G-SS-7044-00

By Dr. Emanuel Hanski, Department of Hormone Research,

The Weizmann Institute of Science, Rehovot, Israel.

"Bordetella Pertussis Invasive Adenylate Cyclase: Its Role in Whooping Cough Pathogenesis and its Possible Use in Development of Acellular Vaccine Against the Disease".

In the first six months of this project our studies were mainly directed at the purification and characterization of the invasive adenylate cyclase of Bordetella pertussis (see objectives 1 and 2 in Attachment 2). As shown in Fig. 1 (and as was indicated in our original Grant Proposal) gel filtration chromatography of dialysed urea extract results in the separation of apparent high (HP) and low (LP) molecular weight peaks of adenylate cyclase (AC) activity. These two peaks which constitute more than 80% of total AC activity found in the extract are devoid of penetration capacity. The peak of fraction inducing cAMP accumulation in target cells (FIC) i.e. the invasive form of the enzyme, migrates between HP and LP and is associated with a minor peak of AC activity. By a two step procedure which includes calmodulin-agarose and hydroxylapatile chromatography we have purified the catalysts of HP and LP (see Table 1). The molecular weights of the HP catalyst is 200 kDa and its specific activity is 466 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, whereas the molecular weight LP catalyst is 47 KDa and its specific activity

1988

is 1685 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. Both catalysts are activated 50-200 fold by calmodulin. However, the half-maximum concentration required for the activation of the HP catalyst ($5.4 \cdot 10^{-9}$M) is 30-fold higher than the one required for the activation of the LP catalyst. Purification of the FIC catalyst encountered difficulties due to enormous variation in this molecular species and due to its minute quantity.

We have raised polyclonal antibodies in guinea pigs against the 47 KDa catalyst isolated from SDS-polyacrylamide gels. As shown in Fig. 2, the serum obtained, efficiently inhibits the AC activity of the purified 47 KDa catalyst. In addition, the polyclonal antibodies specifically recognize on immunoblots, both purified catalysts as well as several other forms of the enzyme of intermediate molecular weight, found in the crude extract (Figs. 3 and 4). The antibody inhibits the activity of all forms of the enzyme in similar titer (Table 2). In addition, the antisera specifically inhibits the penetration of the invasive form into target cells. Taken together, these results suggest that all forms of the B. pertussis AC including the penetrative form are immunologically related and presumably result from a common precursor molecule. What makes FIC invasive? Do the penetration capacity and the catalytic activity reside on the same polypeptide molecule, or is a separate factor required for penetration of the catalyst? These questions are currently under investigation.

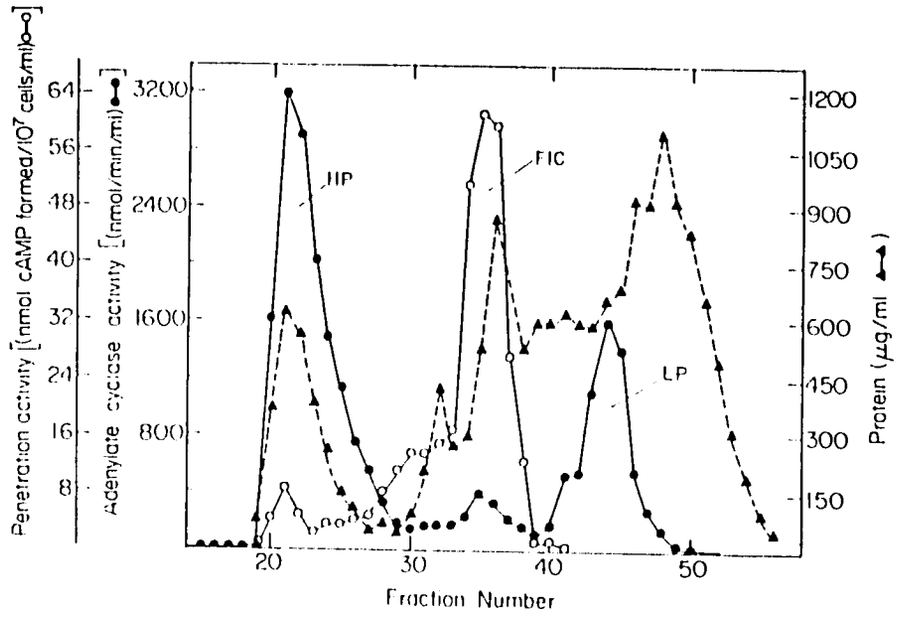

Fig. 1. Gel filtration chromatography of <u>B. pertussis</u> dialyzed urea extract.

Fractions obtained were assayed for: AC activity, ability to induce cAMP accumulation in lymphocytes (penetration activity) and protein.

Step	Protein (mg)	Total activity ($\mu mol \cdot min^{-1}$)	Recovery (%)	Specific activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)
LP pool	97.500	165.0	100	1.7
CaM-agarose	0.090	77.7	47	863
Hydroxylapatite	0.027	45.5	29	1685
HP pool	60.0	307.0	100	5.1
CaM-agarose	0.36	71.4	23	198
Hydroxylapatite	0.09	42.0	14	466

Table 1. Purification of B. pertussis adenylate cyclase from LP and HP.

HP and LP obtained as described in Fig. 1 were subjected to chromatography o calmodulin-agarose and hydroxylapatile. Recoveries are cumulative throug the preparation.

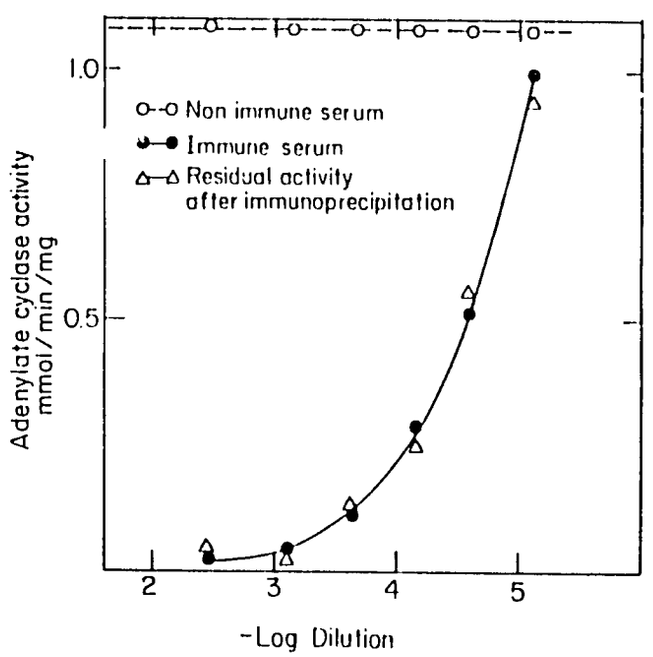

Fig. 2. Inhibition of LP activity be antibodies.

Purified LP 0.04 ng per assay was incubated with the indicated sera dilutions. Aliquots were removed and assayed for AC activity.

200►
120►

47►

1 2 3 4

Fig. 3. Identification of HP and LP on immunoblots.

Purified LP and HP were subjected to SPS-polyacrylamide gel electrophoresis and then electrophoretically transferred to nitrocellulose. The nitrocellulose blots were incubated with immune serum (lane 1,3) or non-immune serum (lanes 2, 4). Visualization of the proteins interacting with antibodies was performed using [^{125}I]-labeled protein A.

-130
-116
-84
-58
-48.5
-36.5
-26.6

Fig. 4. Immunoblotting of dialysed urea extract.

Sample of crude dialyzed extract was electrophoretically transferred to nitrocellulose and visualized as described in Fig. 3. The nitrocellulose blots were incubated with immune (lane 1) or non-immune serum (lane 2).

Adenylate cyclase \ Dilution of immune serum	1:1,000	1:10,000	1:100,000
	Inhibition of cyclase activity (%)		
HP	94.8	82.0	42.5
LP	99.0	88.0	46.0
FIC	96.3	86.6	45.7
B. pertussis extract	94.8	82.4	35.4
Sperm*	0	0	0
Brain, CaM-sensitive*	0	0	0
Brain, CaM-insensitive*	0	0	0

Table 2. The antibody raised against LP inhibits all forms of B. pertussis AC.