Adenylate Cyclase Activity During Phenotypic Variation of *Bordetella pertussis*

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During MgSO_4 -induced modulation of *Bordetella pertussis*, adenylate cyclase activity, histamine-sensitizing activity (HSA) and the major cell-envelope polypeptides with M_r 28000 and 30000 (X polypeptides) were lost synchronously at a rate which could be accounted for by a simple growth-dilution effect. MgSO_4 and other compounds which induced the above phenotypic change caused little inhibition of adenylate cyclase activity. Nicotinic acid was the sole exception and at 4.1 mM-caused 60% inhibition of activity. Lysates of modulated cells, mixed with lysates of unmodulated cells, had no effect on either adenylate cyclase activity or HSA. Protein synthesis was a prerequisite for MgSO_4 -induced modulation and also for the reversal of this process. Exogenous cAMP and dibutyryl cAMP (5 mM) had no counteracting effect on MgSO_4 - or nicotinic acid-induced modulation. The concentration of MgSO_4 required to induce loss of the X polypeptides (10 to 11 mM) was not altered by promoting adenylate cyclase activity by including an activator in the growth medium. In one culture containing 10 mM- MgSO_4 and activator, partial loss of the X polypeptides occurred and yet the extracellular cAMP concentration was twice that of cultures without activator and where full expression of the X polypeptides occurred. [^3H]cAMP-binding activity was detected in cell extracts of several strains of *B. pertussis*, but antiserum against purified *Escherichia coli* catabolite repressor protein gave no reaction with *B. pertussis* cell extracts. Respiration rates with amino acids were similar for modulated and unmodulated variants and an avirulent strain of *B. pertussis*. These results are discussed in relation to a possible causal role for adenylate cyclase in modulation of *B. pertussis*.

INTRODUCTION

Several properties associated with the virulence of *Bordetella pertussis* are lost during the reversible phenotypic change, known as modulation, which occurs when the organism is grown in medium containing one of several salts or nicotinic acid (Lacey, 1960; Pusztai & Joó, 1967; Holt & Spasojevic, 1968; Livey *et al.*, 1978; Dobrogosz *et al.*, 1979; Wardlaw & Parton, 1979; Brownlie *et al.*, 1984). These properties include pertussigen-related activities such as histamine sensitization (see Brownlie *et al.*, 1984), heat-labile toxin, the major cell-envelope polypeptides with M_r 28000 and 30000 (X polypeptides), filamentous haemagglutinin, agglutininogen I, cytochrome d_{629} and adenylate cyclase activity. These properties are also lost during a similar change termed phase degradation, which occurs when *B. pertussis* is repeatedly subcultured *in vitro* (Leslie & Gardner, 1931; Roberts & Ospeck, 1942; Keogh & North, 1948; Eldering *et al.*, 1962; Aprile, 1972; Parton & Wardlaw, 1975; Parton & Durham, 1978; Dobrogosz *et al.*, 1979). Until recently this change was thought to be irreversible (Weiss & Falkow, 1984). The virulent and avirulent phenotypes which the modulation process generates are referred to as X- and C-mode, respectively, whereas the counterpart phenotypes of phase degradation are often referred to as phase I and phase IV, respectively.

Abbreviations: CRP, catabolite repressor protein; HSA, histamine-sensitizing activity.

Adenylate cyclase plays a key regulatory role in the expression of several proteins with diverse functions in the enterobacteria (Pastan & Adhya, 1976; Botsford, 1981), including some associated with virulence (Eisenstein *et al.*, 1981; Martinez-Cadena *et al.*, 1981). Hewlett *et al.* (1979a) and Wardlaw & Parton (1979) suggested that the adenylate cyclase in *B. pertussis* may play a similar regulatory role in the expression of virulence factors. In our accompanying paper (Brownlie *et al.*, 1984), it was shown that loss of adenylate cyclase activity was always accompanied by loss of histamine-sensitizing activity (HSA) and the X polypeptides during modulation that was induced in a variety of ways. The adenylate cyclase of *B. pertussis* is unusual in two respects; it is activated by calmodulin (Goldhammer *et al.*, 1981) and is largely extracytoplasmic in location (Hewlett *et al.*, 1976). The purpose of this investigation was to assess whether or not the loss of adenylate cyclase activity during modulation had a causal or coincidental relationship with the loss of the other properties.

METHODS

Bacterial strains. *Bordetella pertussis* TAB I was isolated pernasally, before the death of an infant and was obtained from Professor G. T. Stewart, Ruchill Hospital, Glasgow, UK. Strain 18334 was a vaccine strain obtained from Connaught Laboratories, Toronto, Canada, and *B. pertussis* D30042 Phase I and D30042 Phase IV were obtained from Dr J. Dolby, Clinical Research Centre, Harrow, London, UK. Strain 18323 was virulent for mice challenged by the intracerebral route and was obtained from Dr F. Sheffield, National Institute of Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB, UK. *Escherichia coli* 1100 HfrH, *thiA*, which expresses wild-type *cya*⁺ and *crp*⁺ characteristics, and its derivative strain 5333 *crp* were obtained from Dr C. R. Harwood, The Medical School, The University, Newcastle upon Tyne, UK. Strains of *B. pertussis* were stored at -70 °C as cell suspensions in a medium consisting of Casamino acids (Difco) (10 g l⁻¹), MgCl₂·6H₂O (0.1 g l⁻¹), NaCl (5 g l⁻¹), CaCl₂ (16 mg l⁻¹), glycerol (10%, v/v), pH 7.1. Strains of *E. coli* were stored at -70 °C as cell suspensions in physiological saline containing 10% (v/v) glycerol.

Growth of organisms. Strains of *B. pertussis* were grown on Bordet-Gengou medium (Gibco-Biocult Diagnostics, Paisley, UK) containing 20% (v/v) defibrinated horse blood (Gibco-Biocult) for 72 h at 35 °C in a moist atmosphere. A loopful of such cultures was used to inoculate 100 ml Stainer & Scholte medium (SS-X; Stainer & Scholte, 1971) contained in 250 ml dimpled flasks. C-mode cells were obtained by growing *B. pertussis* in SS-C medium (SS-X medium with NaCl replaced by 5 g MgSO₄·7H₂O l⁻¹). When X- and C-Hornibrook medium was used, this was made up as described previously (Wardlaw *et al.*, 1976). Commercial bovine catalase is a convenient source of adenylate cyclase activator (calmodulin) and this was present in the medium for those experiments where stated. When *B. pertussis* was grown in medium containing activator, a suspension of bovine liver catalase (10 mg ml⁻¹) was heated at 100 °C for 5 min (activator is heat-stable; Hewlett *et al.*, 1979b), and the supernate was withdrawn, filter sterilized and added to the medium (1%, v/v). Flasks were shaken (80 r.p.m.) at 35 °C for 48 h in an orbital incubator. Strains of *E. coli* were grown on nutrient agar (Oxoid) for 24 h at 37 °C. These cultures were used to inoculate 1 litre amounts of yeast/glucose medium (Pastan *et al.*, 1974) contained in dimpled 2 litre flasks. These were shaken (80 r.p.m.) at 37 °C for 12 h in an orbital incubator.

Cell disruption. When cell lysates were assayed for adenylate cyclase activity, harvested cells were resuspended in saline (2.5 mg protein ml⁻¹) before disruption. When cell lysates together with their culture supernate were assayed for histamine-sensitizing activity (HSA), harvested cells were resuspended to 100 opacity units in their original culture supernate before disruption. Cell lysates were prepared by passing frozen cell suspensions three times through a LKB X-press (Biotech, Stockholm, Sweden).

Adenylate cyclase and cAMP assays. These were assayed as described previously (Brownlie *et al.*, 1984).

cAMP binding activity. Strains of *B. pertussis* were grown in SS-X or SS-C medium for 48 h and *E. coli* strains were grown for 12 h in yeast/glucose medium. Harvested cells were resuspended in 10 mM-Tris/acetate, 1.2 mM potassium acetate, 0.1 mM-dithiothreitol, 25 µM-EDTA, pH 8.2, passed through an X-press three times and centrifuged at 100000 g for 1 h. Supernates were removed and incubated at 4 °C overnight with pancreatic deoxy-ribonuclease (5 µg ml⁻¹) and pancreatic ribonuclease (5 µg ml⁻¹). Samples were assayed for [³H]cAMP binding activity using the method of Pastan *et al.* (1974).

Aerobic catabolism of amino acids. *B. pertussis* was subcultured twice in either SS-X or SS-C medium. Cultures were harvested after growth for 24 h and cells were washed twice in SS buffer (50 mM-Tris/HCl, 43 mM-NaCl, 3.7 mM-KH₂PO₄, 2.7 mM-KCl, 0.5 mM-MgCl₂, 0.2 mM-CaCl₂, pH 7.3). Washed cells were resuspended in SS buffer to 15 mg dry wt ml⁻¹ and incubated at 35 °C for 15 min, to use up endogenous substrates and thereafter at 4 °C. Respiration rates were measured using a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Oh., USA). After determining the endogenous respiration rate, substrate was added to the chamber to give a final concentration of 18 mM.

Histamine-sensitizing activity (HSA). Harvested cells were resuspended in saline to 100 opacity units, heat-killed (56 °C for 30 min) and assayed and evaluated for HSA by the probit method as described by Wardlaw *et al.* (1976). Cell lysates were heat-treated (56 °C for 30 min) and assayed for HSA in only those experiments where stated.

Haemagglutinating activity. Assays were done using microtitre trays. Wells contained 50 µl of bacterial cell suspension (in physiological saline) in a series of doubling dilutions and 50 µl of 2% (v/v) horse red-blood cell suspension. After incubating the trays for several hours at 4 °C, the haemagglutinin titre was recorded as the reciprocal of the highest dilution giving complete haemagglutination.

Slab-gel electrophoresis. Harvested cells were washed once in physiological saline, resuspended to 2 mg protein ml⁻¹ and whole cell proteins were analysed by SDS-PAGE using the methods described previously (Wardlaw *et al.*, 1976; see Brownlie *et al.*, 1984).

Ouchterlony double-diffusion precipitin test. Samples (100 µl) were added to wells (5 mm diameter, 5 mm apart) in gel diffusion medium [15 g purified agar l⁻¹ (Oxoid), 16 g NaCl l⁻¹, 0.12 g methyl orange l⁻¹, 1 g phenol l⁻¹]. Plates were examined after storage in sealed containers at room temperature for several days.

'Crossed-over' immunoelectrophoresis. This was done using the method described by Culliford (1964).

Standardization of cell suspensions. Protein was estimated by the method of Herbert *et al.* (1969) with bovine serum albumin as a standard. For convenience, a standard curve relating dry weight and protein concentration to optical density was used. For HSA determinations, cell suspensions were standardized (before being heat-killed) by comparison with the Fifth International Reference of Opacity (World Health Organization International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Holly Hill, London NW3 6RB, UK).

Chemicals and reagents. Nucleotides and commercial enzymes were from Sigma and tritiated compounds were from Amersham. Goat anti-*E. coli* catabolite repressor protein was obtained from Dr I. Pastan, Laboratory of Molecular Biology, National Cancer Institute, NIH, Bethesda, Md, USA.

RESULTS

Rate of MgSO₄-induced modulation

Adenylate cyclase activity, HSA and the X polypeptides declined rapidly during MgSO₄-induced modulation (Fig. 1). These properties did not change greatly in control experiments where similar inocula were introduced in SS-X medium. It was difficult to assess the rate of loss of HSA accurately due to the large 95% confidence limits obtained (e.g. the confidence limits for HSA potency relative to the inoculum for one experiment were 28–110% at 4 h, 8–30% at 8 h and 4–19% at 20 h). Densitometer traces of SDS-PAGE profiles of whole cells at various stages of modulation revealed not only loss of the X polypeptides (M_r 28000 and 30000) but also loss of at least two other gel bands each with M_r of approximately 100000. These may correspond to the bands with M_r 98000 and 88000 which were reported by Dobrogosz *et al.* (1979) to be lost during modulation. Another minor polypeptide with M_r of approximately 20000 appeared to decrease in intensity during modulation. Loss of adenylate cyclase activity, HSA and the X polypeptides occurred in a synchronous manner and correlated closely with the theoretical loss assuming no synthesis *de novo* and no inactivation or destruction (Fig. 1). This suggests that the loss of these properties could be accounted for by complete repression of the synthesis of adenylate cyclase, pertussigen and the X polypeptides upon transfer of cells to SS-C medium. The extracellular cAMP concentration increased gradually during growth in SS-X medium but remained constant, or declined slightly during growth in SS-C medium (Fig. 2).

Possible involvement of inhibitors in modulation

The low concentration of extracellular cAMP in C-mode cultures (Brownlie *et al.*, 1984) which did not change greatly during growth in SS-C medium (Fig. 2) may have resulted from inhibition of adenylate cyclase activity by pro-C-mode substances. These substances were therefore tested for their effect on adenylate cyclase activity when present in the assay mixture (Table 1). Both nicotinic acid and nicotinamide caused substantial inhibition of enzyme activity (about 60%). Sodium salts, including the pro-X-mode salt NaCl, caused mild inhibition of activity whereas MgSO₄ increased activity slightly.

To test whether SS-C medium inhibited the production of extracellular cAMP, washed X-mode cells were resuspended in SS-X or SS-C medium supplemented with chloramphenicol to

Fig. 1. Loss of adenylate cyclase activity, HSA and the X polypeptides during modulation of *B. pertussis*. SS-C medium (1:25 $\times$ normal strength), temperature equilibrated at 35 °C, received a 20% (v/v) inoculum from a 48 h SS-X culture of strain TAB I. At intervals, cells were harvested, resuspended in saline and assayed for adenylate cyclase activity ($\circ$), heat-treated (56 °C for 30 min) and assayed for HSA ($\bullet$), and washed once in saline and analysed by SDS-PAGE. The intensities of the X polypeptide gel bands ($\square$) were determined by densitometer traces and were standardized against the major gel band, M_r 37 000, which remained relatively constant during growth. The theoretical loss of any property assuming complete repression of synthesis ($\blacksquare$) was calculated using a growth curve based on optical density determinations. Similar data were obtained in a second determination.

Table 1. *Effect of salts and organic acids on adenylate cyclase activity of B. pertussis*

Strain TAB I was grown for 48 h in SS-X medium. Harvested intact cells and cell lysates were assayed for adenylate cyclase activity with various additives present in the assay mixture.

Additive	Final concn (mM)	Pro-X-Mode or pro-C-mode*	Intact cell		Cell lysate	
			Adenylate cyclase activity [pmol cAMP min ⁻¹ (mg protein) ⁻¹]	Relative activity (%)†	Adenylate cyclase activity [pmol cAMP min ⁻¹ (mg protein) ⁻¹]	Relative activity (%)†
None	—	—	960	100	1540	100
NaCl	43	X	755	79	1225	80
MgSO ₄	20	C	995	104	1630	106
Na ₂ SO ₄	43	C	695	72	1165	76
Sodium lactate	86	C	610	64	1185	77
Sodium succinate	86	C	675	70	1155	75
Sodium butyrate	20	C	825	86	1530	99
Sodium caprylate	0.6	C	935	97	1585	103
Nicotinic acid	4.1	C	360	38	605	39
Nicotinamide	4.1	X	340	35	610	40

* See Wardlaw *et al.* (1976) and Brownlie *et al.* (1984).

† Activity in Tricine buffer alone equivalent to 100%.

prevent differential growth, and the supernate was removed at intervals and assayed for cAMP. One set of cell suspensions contained adenylate cyclase activator. The rate of production of extracellular cAMP was the same in both SS-X and SS-C medium, indicating that SS-C did not directly inhibit excretion of cAMP from X-mode cells (Fig. 3). This study also demonstrated that SS-C medium did not interfere with the effect of activator in increasing the production of extracellular cAMP (Fig. 3).

The possibility that a factor was produced during modulation which specifically inactivated X-mode-specific activities was tested by mixing X- and C-mode cell lysates and assaying for adenylate cyclase activity and HSA. For HSA experiments, harvested cells were resuspended to 100 opacity units in culture supernate before cell disruption in case destructive factors were

Fig. 2

Fig. 2. Concentration of extracellular cAMP during growth of *B. pertussis* in X and C medium. SS-X and SS-C medium (1:25 $\times$ normal strength), temperature equilibrated at 35 °C, received a 20% (v/v) inoculum of a 48 h SS-X culture of strain TAB I. Supernate was assayed for cAMP after samples were removed at intervals during growth in SS-X (O) and SS-C (●) medium. Similar data were obtained in a second determination.

Fig. 3

Fig. 3. Effect of MgSO_4 and adenylate cyclase activator on the production of extracellular cAMP by re-suspended X-mode *B. pertussis*. Harvested cells of strain TAB I from a 48 h SS-X culture were washed once and resuspended in medium supplemented with chloramphenicol (100 $\mu\text{g ml}^{-1}$) to give 15 mg dry wt ml^{-1} . Suspensions (10 ml) were incubated in 150 ml flasks in an orbital shaker at 35 °C. At intervals, samples were removed and assayed for cAMP. Media were SS-X (O), SS-X with adenylate cyclase activator (100 $\mu\text{g bovine catalase ml}^{-1}$) (●), SS-C (□), and SS-C with adenylate cyclase activator (■). Results were similar in a second determination.

Table 2. *Adenylate cyclase and HSAs of cell lysates and lysate mixtures of B. pertussis*

For the adenylate cyclase activity experiments, strains were grown in SS-X or SS-C medium for 48 h. For the HSA experiments, strain TAB I was grown in SS-X or SS-C medium for 48 h and strain 18334 was grown in X- and C-mode Hornibrook medium for 48 h. Lysates were prepared as described in Methods, supplemented with chloramphenicol (100 $\mu\text{g ml}^{-1}$), to prevent contamination, and incubated statically at 35 °C for 24 h (24 h growth is normally required for modulation). X- and C-mode cell lysates were mixed in equal volumes; NT, not tested.

Sample	Adenylate cyclase activity [pmol cAMP min ⁻¹ (mg protein) ⁻¹]		HSA potency (%)*	
	Strain		TAB I	18334
X-mode cell lysate (before incubation)		TAB I 1390	950	NT 100
C-mode cell lysate (before incubation)		55	45	NT NT
X-mode cell lysate (after incubation alone)		630	225	100 74 (40, 136)
C-mode cell lysate (after incubation alone)		15	15	<7.5† 6.2 (3.1, 12)
X/C-mode cell lysate mixture (after incubation)		365	105	37 (18, 75) 68 (37, 125)
Expected activity of mixture†		325	120	50 54

* HSA of X-mode cell lysate before incubation in strain 18334, and after incubation in strain TAB I, were taken as 100%. The numbers in parentheses refer to 95% confidence limits.

† The expected activity was calculated assuming no inactivation from C-mode cell lysate (i.e. the means of the individual X- and C-mode cell lysate activities after incubation).

‡ No animals died using 20 opacity units ml^{-1} as highest dose.

released into the medium. Adenylate cyclase activities and HSAs of X- plus C-mode cell lysate mixtures were similar to those expected assuming that no destructive factors were present (Table 2). This was also the case for the HSA of an X- plus C-mode cell lysate mixture of strain 18334

Table 3. *Effect of chloramphenicol on C- and X-modulation of B. pertussis*

Media received a 10% (v/v) inoculum from either a 48 h SS-X or SS-C culture. After 48 h incubation at 35 °C harvested cells were assayed for intact-cell adenylate cyclase activity, HSA and the X polypeptides. Chloramphenicol was present at 100 µg ml⁻¹. The adenylate cyclase activities shown are the means of two determinations; +, present; -, absent.

Inoculum	Medium	Chloramphenicol	HSA potency (%) [*]	Adenylate cyclase activity [pmol cAMP min ⁻¹ (mg protein) ⁻¹]	X polypeptides
SS-X-grown cells	SS-X	{ -	100	990	+
		{ +	93 (50, 176)	660	+
	SS-C	{ -	6 (2, 13)	15	-
		{ +	100 (53, 189)	625	+
SS-C-grown cells	SS-X	{ -	100	915	+
		{ +	1.9 (0.0, 6.9)	10	-
	SS-C	{ -	<4.2†	<5‡	-
		{ +	1.3 (0.0, 5.5)	15	-

^{*} HSA potency of SS-X- (without chloramphenicol) grown cells was taken as 100%. The numbers in parentheses refer to 95% confidence limits.

† No animals died using 20 opacity units ml⁻¹ per mouse as highest dose.

‡ Limit of detection.

Table 4. *Critical concentration of MgSO₄ required to induce loss of the X polypeptides*

Strain TAB I was grown for 48 h in SS-X medium where the NaCl was proportionately replaced by MgSO₄. Supernate of a heat-treated (100 °C for 5 min) bovine catalase solution (10 mg ml⁻¹; 1% v/v) was added as a source of adenylate cyclase activator. Harvested cells were analysed by SDS-PAGE to determine whether the X polypeptides were present (X) or absent (C). 'I' refers to an intermediate variant where the X polypeptides were partially reduced. Culture supernate was assayed for cAMP (numbers in parentheses are pmol cAMP ml⁻¹); NT, not tested.

MgSO ₄ (mm) in medium ...		0	8	9	10	11	12	20
NaCl (mm) in medium ...		43	26	24	22	19	17	0
		Expt no.						
Bovine catalase absent	{ 1	X (110)	X (125)	NT	X (110)	NT	C (<5)	C (<5)
	{ 2	X (120)	X (120)	X (80)	X (110)	C (<5)	C (<5)	C (<5)
	{ 3	X (125)	X (95)	X (140)	X (95)	C (<5)	C (<5)	C (<5)
Bovine catalase present	{ 1	X (565)	X (490)	X (540)	I (210)	C (<5)	C (<5)	C (<5)
	{ 2	X (510)	X (535)	X (565)	X (520)	C (<5)	C (<5)	C (<5)
	{ 3	X (535)	X (580)	X (530)	X (545)	C (<5)	C (<5)	C (<5)

after growth in X- or C-mode Hornibrook medium. This strain and medium were used by Idigbe *et al.* (1981) who found HSA to be lost much more rapidly during modulation than could be accounted for by a simple dilution effect, suggesting selective destruction.

Requirement for protein synthesis during modulation

The effect of chloramphenicol on MgSO₄-induced loss of adenylate cyclase activity, HSA and the X polypeptides was investigated. Strain TAB I was incubated in SS-X or SS-C medium with and without chloramphenicol (100 µg ml⁻¹). This concentration of antibiotic completely inhibited growth and incorporation of L-[³H]methionine into acid-insoluble material (data not shown). Inoculation of SS-X-grown cells into SS-C medium containing chloramphenicol resulted in a drop of only 30% in adenylate cyclase activity after 48 h, a value similar to that obtained from cells in SS-X medium with added antibiotic (Table 3). Growth in SS-C medium alone, on the other hand, promoted substantial loss of activity (98%). cAMP in the medium did not increase substantially under conditions which did not allow bacterial growth, i.e. in medium containing chloramphenicol (data not shown). Similarly, prolonged exposure to SS-X or SS-C

medium containing chloramphenicol caused no significant loss of HSA or the X polypeptides (Table 3). Growth in SS-C medium after inoculation with SS-X grown cells decreased HSA by 94% and caused complete loss of the X polypeptides. It can be concluded that protein synthesis is a requirement for the loss of all these properties and that incubation in SS-C medium does not bring about the destruction of these properties. The effect of chloramphenicol on C to X modulation was also investigated and, as might have been expected, the expression of adenylate cyclase activity, HSA and the X polypeptides also required protein synthesis (Table 3).

Effect of added cyclic nucleotides

Addition of either cAMP (5 mM) or N⁶,O^{2'}-dibutyryl cAMP (5 mM) to the medium had no effect in counteracting MgSO₄-induced loss of adenylate cyclase activity, the X polypeptides and haemagglutinating activity, or nicotinic acid-induced loss of adenylate cyclase activity and the X polypeptides. Although it has not been shown conclusively that the nucleotides are taken up by the cells, preliminary experiments did show the incorporation of [³H]cAMP and [³H]dibutyryl cAMP into filtered X-mode cells after incubation of the cells in SS-X or SS-C medium at rates between 0.4–0.6 nmol min⁻¹ (mg dry wt)⁻¹.

Attempts to manipulate adenylate cyclase activity

Hewlett & Wolff (1976) reported that NaF strongly inhibited activity of purified *B. pertussis* adenylate cyclase. This was confirmed in the present study where 40 mM-NaF in the assay mixture inhibited adenylate cyclase activity of cell-lysate by 90%. However, although the presence of NaF (40 mM) in SS-X medium partially inhibited growth it did not alter production of extracellular cAMP or the X polypeptides. This may be because *B. pertussis* cells might not be very permeable to NaF.

Commercial bovine catalase is a convenient source of adenylate cyclase activator in *B. pertussis* (Hewlett *et al.*, 1978). It was thought that this might be a useful tool for assessing the role that cAMP might play in modulation. Experiments showed that activator had little effect on the production of HSA, the X polypeptides or extracellular cAMP after 48 h growth in SS-C medium. When the activator was present in SS-X medium it raised production of extracellular cAMP approximately fivefold, but had no noticeable effect on HSA or the X polypeptides after 48 h growth. Thus it appeared that modulation conditions in SS-C medium were too severe to increase cAMP production to a detectable level, even with activator in the medium. Lacey (1960) reported that an intermediate (I) mode arose when *B. pertussis* was grown with a critical ratio of pro-X- to pro-C-mode salts. If cAMP played a role in modulation it was thought possible that the addition of activator to the medium might shift the critical concentration of MgSO₄ required to induce modulation. Strain TAB I was grown for 48 h in a series of modified SS-X media where the NaCl was progressively replaced by MgSO₄. A parallel series of cultures contained activator. The critical concentration of MgSO₄ required to induce modulation, as judged by the loss of the X polypeptides, was 11 mM (Table 4) and this was not altered by the presence of the activator. Using batch cultures and increments of 1 mM-MgSO₄, it was difficult to obtain an I-mode variant showing partial loss of the X polypeptides. In six series of MgSO₄/NaCl concentrations, an I-mode was obtained once only, suggesting that this variation to I-mode is critically dependent on the concentration of MgSO₄. Extracellular cAMP was detectable only in those cultures where the X polypeptides were present. In cultures with activator, cAMP concentrations ranged from 490 to 580 pmol ml⁻¹ while in cultures without activator, the range was 80 to 140 pmol ml⁻¹. The one case where an I-mode variant was obtained (where the X polypeptides were partially lost) was in a culture grown with activator. The extracellular cAMP concentration in this culture was 210 pmol ml⁻¹, which was about twice that found in X-mode cultures without activator.

cAMP binding activity

cAMP regulates gene transcription in *E. coli* by binding to a cytoplasmic protein termed the catabolite repressor protein (CRP). This complex (cAMP–CRP) selectively activates or inhibits gene expression (Botsford, 1981). If cAMP plays a regulatory role in gene expression in *B.*

Table 5. [^3H]cAMP binding activity in extracts of *E. coli* and *B. pertussis*

E. coli strains were grown for 12 h in yeast/glucose medium and *B. pertussis* strains were grown for 48 h in SS-X medium. Supernates of cell lysates were assayed for [^3H]cAMP binding activity.

Bacterial strain	Expt no. . . .	cAMP binding activity [pmol [^3H]cAMP bound (mg protein) $^{-1}$]		
		1	2	3
<i>E. coli</i> 1100		6.9	4.7	2.9
<i>E. coli</i> 5336 <i>crp</i>		0.4	0.0	1.1
<i>B. pertussis</i> TAB I		3.1	1.8	3.8
<i>B. pertussis</i> TAB I (C-mode)*		2.2		
<i>B. pertussis</i> 18323		2.7		
<i>B. pertussis</i> D30042I		1.8		
<i>B. pertussis</i> D30042IV		1.6		

* Grown for 48 h in SS-C medium.

Table 6. Respiration rate of *B. pertussis* variants in the presence of different amino acids

Strain D30042I was subcultured twice for 24 h in either SS-X or SS-C medium and strain D30042IV was subcultured twice for 24 h in SS-X medium. Respiration rates were determined at 35 °C for twice washed cells using a Clark oxygen electrode. Cells were present at 0.5 mg dry wt ml $^{-1}$ and substrate at 18 mM. Substrate respiration rate was corrected for endogenous rate. Results are the means of three determinations $\pm$ SD.

Amino acid	Respiration rate [$\mu\text{l O}_2 \text{ min}^{-1} (\text{mg dry wt})^{-1}$]		
	X-mode	C-mode	Phase IV
L-Glutamate	6.3 $\pm$ 0.9	6.5 $\pm$ 0.6	6.5 $\pm$ 0.3
L-Aspartate	5.6 $\pm$ 0.8	7.2 $\pm$ 1.3	6.5 $\pm$ 0.2
L-Proline	3.5 $\pm$ 0.6	3.9 $\pm$ 0.2	3.4 $\pm$ 0.2
L-Serine	2.5 $\pm$ 0.3	2.9 $\pm$ 0.2	2.5 $\pm$ 0.3
L-Alanine	2.2 $\pm$ 0.3	2.4 $\pm$ 0.1	2.7 $\pm$ 0.3
Endogenous rate	0.2 $\pm$ 0.1	0.3 $\pm$ 0.2	0.2 $\pm$ 0.1

pertussis, it is likely that the organism possesses a protein analogous to the CRP in *E. coli*. [^3H]cAMP binding activity was demonstrated in extracts of strains of *E. coli* and *B. pertussis* (Table 5). Low activity was detected in *E. coli crp* (a strain lacking a functional CRP) and binding activity in *B. pertussis* strains was midway between this strain and the wild type-*E. coli* Crp $^+$. No significant differences were apparent between X- and C-mode cells or between phase I and phase IV variants. The possible presence in *B. pertussis* of a protein immunologically similar to *E. coli* CRP was tested by Ouchterlony double-diffusion precipitin and 'crossed-over' immunoelectrophoresis tests using goat anti-*E. coli* CRP serum. In both tests, one strong and one faint precipitin line were observed against a cytoplasmic fraction of *E. coli* Crp $^+$ but none was obtained against cytoplasmic fractions of *B. pertussis* TAB I or 18334, even at twice the protein concentration of the *E. coli* fraction.

Aerobic catabolism of amino acids

The respiration rates of *B. pertussis* variants in the presence of various amino acids were investigated to see if cAMP played a role in regulating the expression of catabolic functions in this organism as it does in the enterobacteria (Pastan & Adhya, 1976). Washed suspensions of *B. pertussis* catabolized several amino acids at significant rates, as indicated by the utilization of O $_2$ (Table 6). Increased respiration was not detected with D-ribose, D-glucose, D-sucrose, glycine, L-glutamine, L-arginine, L-lysine, L-threonine, L-histidine or citrate. While significant differences existed between the respiration rates in the presence of different amino acids, there were no obvious differences between the respiration rates by different variants in the presence of any particular amino acid. X- and C-mode variants of *B. pertussis* D30042I also utilized acetate, pyruvate, lactate, succinate and formate at rates which were similar for both variants (data not shown).

DISCUSSION

From this study, two lines of evidence indicate that adenylate cyclase is not the target site for modulation and that low concentrations of cAMP are due to repression of adenylate cyclase synthesis, itself subject to the regulatory mechanism perturbed during modulation. Firstly, loss of intact-cell adenylate cyclase activity during MgSO_4 -induced modulation could be accounted for by complete repression of adenylate cyclase synthesis. Secondly, no evidence favouring direct or indirect inhibition of adenylate cyclase activity, or an inactivating mechanism, which might have occurred during modulation was obtained. Also, it is unlikely that cAMP plays a secondary role in modulation by repressing synthesis of pertussigen and the X polypeptides since: (a) loss of intact-cell adenylate cyclase activity during MgSO_4 -induced modulation did not precede but paralleled the loss of HSA and the X polypeptides; (b) after growth of *B. pertussis* in modified SS-X medium containing 10 mM- MgSO_4 and calmodulin, partial repression of the X polypeptides occurred, yet extracellular concentrations of cAMP were twice as high as those in normal SS-X cultures; (c) exogenous cAMP or dibutyryl cAMP in the growth medium had no observable effect in counteracting modulation.

The time-course of MgSO_4 -induced modulation revealed that loss of adenylate cyclase activity, HSA and the X polypeptides occurred synchronously and could be accounted for by an immediate and complete repression of synthesis of the responsible components. This is consistent with results reported by Hall *et al.* (1982) who investigated the rate of loss of adenylate cyclase activity and the X polypeptides during MgSO_4 -induced modulation. However, in an analysis of six independent experiments, Idigbe *et al.* (1981) reported that strain 18334, grown in modified Hornibrook medium containing MgSO_4 , underwent a more rapid loss of pertussigen-associated activities, heat-labile toxin and the X polypeptides than could be accounted for by a simple growth-dilution effect. These workers suggested that selective destruction of X-mode components took place during modulation. However, they also observed a much slower growth rate (mean generation time 6 to 7 h) than that observed in the present study (mean generation time 3.2 h). This difference in the growth rate and the use of different strains and media may explain the difference in results from those reported here. Recently, Robinson *et al.* (1983) studied the rate of modulation of *B. pertussis* in continuous culture. These workers also noted that the X- and C-mode transition took place faster than the theoretical washout rate, again suggesting selective destruction of X-mode properties. Selective destruction may be noticeable only with a slow growth rate.

There was no evidence for destructive or inactivation factors for pertussigen or adenylate cyclase when X- and C-mode cell lysates were mixed together. However, moderate destruction or inactivation of pertussigen might not be noticed due to the large 95% confidence limits obtained for the HSA tests. It is also possible that destructive mechanisms require the integrity of intact cells or are extremely labile. Synthesis of selectively destructive or inactivating factors would be a rather inefficient way to control the physiology of an organism, but it is not known if modulation has a real counterpart in nature.

Previous studies have shown that extracellular concentrations of cAMP are much lower after growth in C media than after growth in X media (Parton & Durham, 1978; Hall *et al.*, 1982; Brownlie *et al.*, 1984). Low concentrations of extracellular cAMP after growth in C media cannot be accounted for by direct inhibition of adenylate cyclase activity by pro-C-mode salts, as most of these caused no more inhibition than did the pro-X-mode salt NaCl. The moderate inhibition caused by NaCl, Na_2SO_4 , sodium succinate and sodium butyrate was presumably due to Na^+ ions which Hewlett & Wolff (1976) reported to cause mild inhibition of adenylate cyclase activity. The inhibition caused by nicotinic acid and nicotinamide was more marked (about 60% loss of activity). Growth in SS-X medium containing nicotinic acid resulted in extracellular concentrations of cAMP less than 5% of those after growth in normal SS-X medium (Brownlie *et al.*, 1984). It is unlikely that the observed 60% inhibition of adenylate cyclase by nicotinic acid fully accounts for this. Nicotinic acid-induced modulation is probably not due to nicotinic acid inhibition of adenylate cyclase activity since nicotinamide, which also caused 60% inhibition of adenylate cyclase activity, does not induce modulation (Wardlaw *et al.*, 1976). Differences in the amounts of extracellular cAMP in X- and C-mode cultures cannot be attributed to differences in

energy charge as intracellular pools of ATP are similar for X- and C-mode cells (Ezzell *et al.*, 1981).

After incubation of X-mode *B. pertussis* in SS-X and SS-C medium supplemented with chloramphenicol, the specific activity of intact-cell adenylate cyclase was about 60 to 80% of that after growth in SS-X medium, whereas after growth in SS-C medium, activity was about 1.0 to 2.5% of that after growth in SS-X. This observation suggests that adenylate cyclase is a relatively stable enzyme and that the loss of activity observed during modulation is not due to direct inactivation by prolonged exposure to the medium, but requires growth or protein synthesis. Similarly, loss of HSA and the X polypeptides during modulation required growth or protein synthesis. Chloramphenicol also prevented the bacteria regaining these properties during C to X modulation, presumably because the proteins have to be synthesized *de novo* as opposed to being reactivated.

So far as is known, all cAMP regulatory systems in bacteria require a functional CRP although cAMP-binding proteins have been identified in only a few bacteria, e.g. *E. coli*, *Salmonella typhimurium*, *Caulobacter crescentus*, *Myxococcus xanthus*, *Beneckea harveyi* and *Alcaligenes eutrophus* (Pastan & Adhya, 1976; Shapiro, 1976; Orłowski, 1980; Nealson & Hastings, 1979; Tait *et al.*, 1981). In this study, [³H]cAMP binding activity was detected in several strains of *B. pertussis* and, on average, was approximately half that obtained from the wild-type *E. coli* Crp⁺ and approximately four times that obtained for *E. coli* *crp*. It is not known whether this binding activity is attributable to a protein with a real physiological function. Anti-*E. coli* CRP gave a clear precipitin reaction with a cytoplasmic *E. coli* fraction, but gave no reaction with *B. pertussis* cytoplasmic fractions. Using this same antiserum, Ouchterlony double-diffusion studies showed cross-reactivity between purified *E. coli* CRP and cell extracts of *E. coli*, *Proteus mirabilis*, *Photobacterium fischeri*, *Klebsiella aerogenes*, *S. typhimurium* and *Caulobacter crescentus* (Anderson & Pastan, 1973). The CRP of *Beneckea harveyi* is also immunologically homologous with that of *E. coli* (Nealson & Hastings, 1979). To the authors' knowledge, there have been no reports of a CRP which does not react with anti-*E. coli* CRP.

Of 11 amino acids tested, washed suspensions of *B. pertussis* aerobically catabolized only L-glutamate, L-aspartate, L-proline, L-serine and L-alanine at detectable rates. This is consistent with the findings of Jebb & Tomlinson (1951). While significant differences existed between respiration rates in the presence of different amino acids, the respiration rates of the variants were similar with any particular amino acid. The fact that the C-mode and phase IV variants produced lower concentrations of cAMP during growth than did the X-mode variant suggests that cAMP was not involved in the regulation of the permeases or enzymes required for amino acid utilization. It also indicates that the mechanisms of modulation and phase degradation do not alter uptake of amino acids.

Three observations suggest that during the course of evolution adenylate cyclase may have adopted different physiological roles in *E. coli* and *B. pertussis*. (a) A large proportion of the adenylate cyclase activity in *B. pertussis* is extracytoplasmic or is secreted into the medium. (b) *B. pertussis* adenylate cyclase shows a marked responsiveness to calmodulin. (c) A protein immunologically similar to *E. coli* CRP could not be detected in *B. pertussis*. This may be related to the differences in physiology of the two organisms: *E. coli* is a facultative anaerobe which utilizes sugars preferentially as sources of energy whereas *B. pertussis* is a strict aerobe which utilizes only amino acids as sources of energy.

It has been suggested that adenylate cyclase in *B. pertussis* plays a role in virulence by altering cAMP-related functions in the host. Confer & Eaton (1982) reported that after incubating neutrophils with *B. pertussis* extracts, several functions of the neutrophils were altered and the intracellular amount of cAMP in the neutrophils was higher than reported previously. In addition, the neutrophils had a high adenylate cyclase activity, which because of its heat stability (100 °C for 5 min), was attributed to the presence of the *B. pertussis* enzyme. In the present study, however, 100 °C for 10 min destroyed all detectable adenylate cyclase activity in *B. pertussis* intact cells. Hewlett & Wolff (1976) also reported that purified adenylate cyclase from *B. pertussis* lost 90% of its activity after 20 min at 56 °C. Clearly, further investigation of the effects of *B. pertussis* adenylate cyclase on neutrophil function is needed.

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