Secretion of the *Bordetella pertussis* Adenylate Cyclase from *Escherichia coli* Containing the Hemolysin Operon†

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ABSTRACT: The extracellular calmodulin-sensitive adenylate cyclase produced by *Bordetella pertussis* is synthesized as a 215-kDa precursor. This polypeptide is transported to the outer membrane of the bacteria where it is proteolytically processed to a 45-kDa catalytic subunit which is released into the culture supernatant [Masure, H. R., & Storm, D. R. (1989) *Biochemistry 28*, 438-442]. The gene encoding this enzyme, *cya*A, is part of the *cya* operon that also includes the genes *cya*B, *cya*D, and *cya*E. A comparison of the predicted amino acid sequences encoded by *cya*A, *cya*B, and *cya*D with the amino acid sequences encoded by *hly*A, *hly*B, and *hly*D genes from the hemolysin (*hly*) operon from *Escherichia coli* shows a large degree of sequence similarity [Glaser, P., Sakamoto, H., Bellalou, J., Ullmann, A., & Danchin, A. (1988) *EMBO J. 7*, 3997-4004]. Complementation studies have shown that *Hly*B and *Hly*D are responsible for the secretion of *Hly*A (hemolysin) from *E. coli*. The signal sequence responsible for secretion of hemolysin has been shown to reside in its C-terminal 27 amino acids. Similarly, *Cya*B, *Cya*D, and *Cya*E are required for the secretion of *Cya*A from *Bordetella pertussis*. We placed the *cya*A gene and a truncated *cya*A gene that lacks the nucleotides that code for a putative C-terminal secretory signal sequence under the control of the *lac* promoter in the plasmid pUC-19. These plasmids were transformed into strains of *E. coli* which contained the *hly* operon. The truncated *cya*A gene product, lacking the putative signal sequence, was not secreted but accumulated inside the cell. However, induction of the full-length *cya*A gene in these strains led to cell-associated and secreted adenylate cyclase activity. In the absence of the *hly* operon, there was no adenylate cyclase activity secreted from *E. coli* containing *cya*A. The secreted adenylate cyclase had a molecular weight of 215K while the cell-associated activity corresponded to polypeptides of 215, 103, and 51 kDa. Partially purified preparations of the extracellular adenylate cyclase secreted by *E. coli* did not elevate cAMP levels in mouse neuroblastoma cells. These data indicate that the gene products of the *hly* operon recognized the signal sequence of *cya*A and facilitated the secretion of the 215-kDa precursor of *B. pertussis* adenylate cyclase from *E. coli*.

Bordetella pertussis, the causative agent of whooping cough, produces several virulence factors implicated in the pathogenesis of the disease (Weiss & Hewlett, 1986; Wardlaw & Parton, 1988). One of these factors is a calmodulin-sensitive adenylate cyclase that is synthesized as a 215-kDa precursor and transported to the outer membrane of the bacteria. This precursor is proteolytically processed, and a 45-kDa catalytic subunit from the N terminus is released into the culture media (Masure & Storm, 1989). Preparations of this form of the extracellular enzyme elevate intracellular cAMP levels when incubated with a wide variety of eucaryotic cells (Shattuck & Storm, 1985; Selfe et al., 1986; Masure et al., 1988). Recently, the gene (*cya*A) encoding this protein has been shown to be part of an operon (*cya*) consisting of additional genes designated *cya*B, *cya*D, and *cya*E. Complementation studies have shown that *cya*B, *cya*D, and *cya*E are required for the secretion of the adenylate cyclase from *B. pertussis* (Glaser et al., 1988b).

Many strains of *Escherichia coli* associated with extraintestinal infections release a protein, hemolysin, into the culture media that is thought to play a role in the pathogenicity of these organisms (Brooks et al., 1980; Evans et al., 1981; Welch et al., 1981). Hemolysin is a 107-kDa protein that lyses red blood cells (Felmlee et al., 1985b; Bhakdi et al., 1986; Felmlee & Welch, 1988). The gene encoding hemolysin has been cloned and sequenced. Like the adenylate cyclase from *B. pertussis*, the *hly*A structural gene is part of an operon composed of genes designated *hly*C, *hly*B, and *hly*D (Felmlee et al., 1985b). Complementation studies have shown that *Hly*B and *Hly*D are required for the secretion of *Hly*A (Goebel & Hedgepeth, 1982; Mackman & Holland, 1984; Nicaud et al., 1985a,b; Mackman et al., 1986). Furthermore, the C-terminal domain of hemolysin has been shown to contain a signal sequence required for secretion of the protein (Nicaud et al., 1986; Mackman et al., 1987; Koronakis et al., 1989).

Comparison of the predicted amino acid sequences of *Cya*A, *Cya*B, and *Cya*D with the predicted sequences of *Hly*A, *Hly*B, and *Hly*D shows 25%, >50%, and 32% overall sequence similarity, respectively (Glaser et al., 1988b). Since the *cya*B, *cya*D, and *cya*E genes and the *hly*B and *hly*D genes are required for the secretion of the two toxins, we hypothesized that the gene products of the *hly* operon responsible for the secretion of hemolysin might promote the secretion of *Cya*A from *E. coli*.

In this study, we placed the *cya*A gene under the control of the *lac* promoter in a pUC expression vector. Strains of *E. coli* that harbored both the *cya*A expression vector and a second plasmid containing the hemolysin operon were examined for the secretion of adenylate cyclase activity. The data

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reported in this paper suggest a similar molecular mechanism for the secretion of the adenylate cyclase from *B. pertussis* and hemolysin from *E. coli*.

Experimental Procedures

Materials. *E. coli* strains DH1 (Low, 1968) and JG1 (Leib & Gerlt, 1983) have been described previously. WAM 582, a DH1 derivative that harbors the plasmid pSF4000 which contains the hemolysin operon, was a gift from Dr. R. M. Welch (Welch et al., 1983). The calmodulin (CaM)[1] expression vector pVUC-1 (Roberts et al., 1985) was a gift from Dr. D. M. Watterson. Cloning vector pUC-19 was from Pharmacia.

General Recombinant DNA Techniques. Large-scale isolation of plasmid DNA, minipreparation of plasmid DNA, restriction enzyme digestion, ligation, and transformation were performed as described elsewhere (Silhavy et al., 1984; Maniatis et al., 1982).

Isolation of Constructs of the B. pertussis Adenylate Cyclase Gene. The gene encoding the calmodulin-sensitive adenylate cyclase (*cya*A) was isolated from *B. pertussis* by the method of Glaser et al. (1988a) with some modifications as described previously by our laboratory (Au et al., 1989). The clone pDA2, a pUC-19 derivative, contains an 8.2-kb fragment of the *cya* operon. It includes the *cya*A and *cya*B genes and part of the *cya*D gene. A *Hin*dIII digest of this plasmid was partially digested with *Bsm*I and yielded two fragments. A 5.5-kb fragment contained the entire *cya*A gene and the first 207 nucleotides of the *cya*B gene. A 5.1-kb fragment contained a truncated *cya*A gene with 223 nucleotides removed from the 3′ end. This encoded *cya*A lacking the final 75 amino acids from the C terminus. Blunt ends were made on these fragments with the Klenow fragment of DNA polymerase I. The fragments were then cut with *Bam*HI and ligated into the *Bam*HI–*Sma*I sites of pUC-19. These plasmids were cloned into a cya$^-$ strain of *E. coli* (JG-1) and plated on McConkey agar plates. The production of cAMP by the plasmid-encoded adenylate cyclase enabled the bacteria to ferment lactose. Therefore, correct inserts of the *cya*A gene into cloning vectors produced blue colonies on McConkey agar plates. Other studies have suggested that this complementation requires the presence of the gene for calmodulin, an activator of the *B. pertussis* adenylate cyclase (Glaser et al., 1988a). However, the high copy number of pUC-19 produced enough basal adenylate cyclase activity that coexpression of calmodulin was not required. Insertion of the *cya*A gene into these clones was confirmed by restriction endonuclease mapping as well as by adenylate cyclase assay of cell lysates. Two plasmids were isolated that expressed adenylate cyclase activity under the control of the *lac* promoter (pRM003, 5.5-kb insert, full-length *cya*A; pRM005, 5.1-kb insert, truncated *cya*A).

Construction of E. coli Strains. The plasmids pRM003 and pRM005 contain ampicillin resistance gene as a selectable marker. These plasmids were transformed individually into either *E. coli* strain DH1 or WAM582 which is a DH1 derivative that contains the plasmid pSF4000. The pSF4000 plasmid contains the hemolysin operon under the control of its own promoter and encodes chloramphenicol resistance as a selectable marker. Transformants harboring either pRM003 and pSF4000 (HAC-1) or pRM005 and pSF4000 (HtAC-1) were selected on solid LB medium with 50 μg/mL ampicillin and 12.5 μg/mL chloramphenicol. Transformants of DH1 containing only pRM003 or pRM005 (AC-1 and tAC-1, respectively) were selected on solid LB medium with 50 μg/mL ampicillin.

Expression and Secretion of the B. pertussis Adenylate Cyclase in E. coli. Different strains of *E. coli* were grown at 37 °C in LB medium containing the appropriate antibiotics (Miller, 1972). When cultures reached the mid-log phase of growth (OD_{650}, 0.2–0.3), IPTG was added to a final concentration of 1 mM, and incubation was continued. Samples (4 mL) were removed at various time points, and bacterial cells were collected by centrifugation at 8000*g* for 20 min. The culture supernatants were saved for assay of adenylate cyclase activity and for SDS–polyacrylamide gel electrophoresis. The cell pellet was suspended in 0.5 mL of buffer containing 20 mM Tris-HCl, pH 7.5, 40 mM NaCl, and 5 mM $MgCl_2$. The cells were disrupted with a Branson sonifier cell disrupter for 10 s with the power setting at 50% of maximum. This suspension was spun in a microcentrifuge for 5 min. The supernatants which contained the cellular extracts were also saved for assay of adenylate cyclase activity and SDS–polyacrylamide gel electrophoresis. Samples were quick-frozen and stored at −80 °C.

Partial Purification of the B. pertussis Adenylate Cyclase Secreted from E. coli. An *E. coli* strain that secreted the *B. pertussis* adenylate cyclase was grown to an OD_{650} of 1.2 in LB media with appropriate antibiotics. Twenty-five milliliters of the cell suspension was pelleted by centrifugation at 5000*g* for 30 min and resuspended in an equal volume of M63 media supplemented with 20 mg/mL casamino acids and 1 mM $CaCl_2$ (Silhavy et al., 1984). Two milliliters of this suspension was added to 1.5 L of the supplemented M63 media. Eighteen liters of bacteria was grown in a rotary shaker at 37 °C until they reached an OD_{650} of 0.4 and were then adjusted to 1 mM IPTG. The bacteria were grown for another 6 h to an OD_{650} of 1.2. The culture supernatant was isolated by centrifugation at 5000*g* for 30 min. Adenylate cyclase activity was partially purified from the culture supernatant by QAE anion-exchange chromatography as described previously (Shattuck et al., 1985; Masure et al., 1988). Column fractions were pooled, concentrated with an Amicon PM-10 membrane, and stored at −80 °C. The specific activity of this enzyme preparation was 3.3 μmol of cAMP min^{-1} mg^{-1}.

Adenylate Cyclase Assay. The production of cAMP was measured by the method of Salomon et al. (1974). Each assay contained 20 mM Tris-HCl (pH 7.4), 1 mM [α-^{32}P]ATP, 5 mM $MgCl_2$, 1 mM EDTA, and 0.1% BSA. Some samples were assayed in the presence of 2.4 μM calmodulin. Calmodulin was prepared from bovine brain by the procedure of Masure et al. (1984). One unit of adenylate cyclase catalyzed the synthesis of 1 nmol of cAMP/min.

Determination of Intracellular cAMP Levels in Neuroblastoma Cells. Intracellular levels of cAMP in mouse neuroblastoma cells (N1E-115) exposed to different bacterial preparations were measured as described previously (Masure et al., 1988).

SDS–Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed according to Laemmli (1971). Recovery of adenylate cyclase activity from SDS–polyacrylamide gels was performed as described previously (Masure & Storm, 1989).

Results

Expression and Secretion of the B. pertussis Adenylate Cyclase from E. coli. A summary of the various bacterial strains and plasmids used in this study is reported in Table

[1] Abbreviations: BSA, bovine serum albumin; CaM, calmodulin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; cfu, colony forming unit; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

Table I: Bacterial Strains and Plasmids Used in This Study

strain designation	plasmids	comments	reference
WAM582	pSF4000	pACYC derivative with an 11-kb insert of *hly* operon	Welch et al. (1983)
AC-I	pRM003	pUC-19 derivative with a 5.5-kb insert of *cya*A gene	this study
tAC-1	pRM005	pUC-19 plasmid with the 5.1-kb insert of truncated *cya*A gene	this study
HAC-1	pRM003/ pSF4000	*cya*A gene and *hly* operon	this study
HtAC-1	pRM004/ pSF4000	truncated *cya*A gene and *hly* operon	this study
HAC-2	pDA2/ pSF4000	*cya*A, *cya*B, and truncated *cya*D genes and *hly* operon	Au et al. (1989), this study

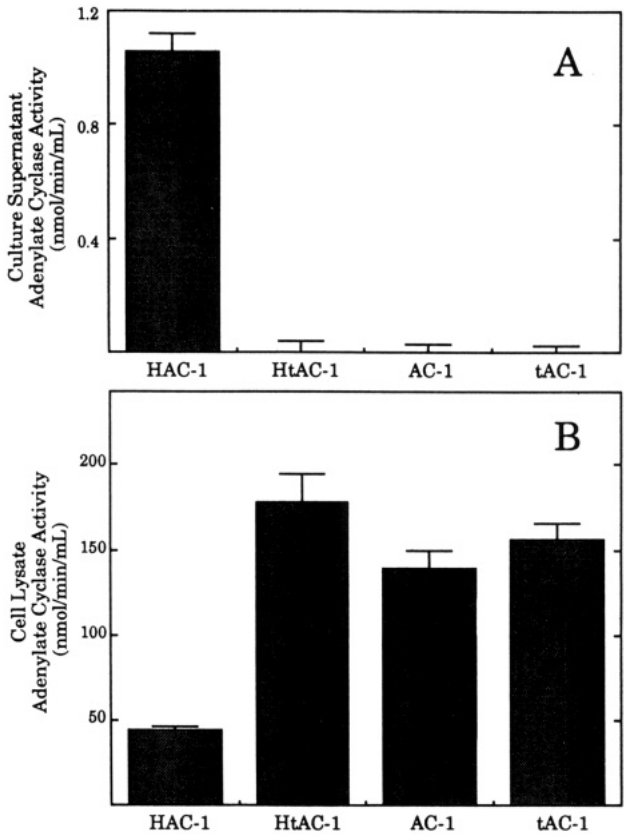

FIGURE 1: Expression and localization of the calmodulin-sensitive adenylate cyclase from *B. pertussis* in *E. coli*. Strains of *E. coli* harboring the plasmid-encoded full-length *cya*A gene with (HAC-1) or without (AC-1) the *hly* operon and strains harboring the plasmid-encoded truncated *cya*A gene with (HtAC-1) or without (tAC-1) the *hly* operon were grown to early log phase, and the expression of the *cya*A gene was induced with 1 mM IPTG. The bacteria were harvested at late log phase and the culture supernatant (A) and cell lysate (B) isolated and assayed for adenylate cyclase activity.

I. HAC-1, HtAC-1, AC-1, and tAC-1 strains of *E. coli* were grown in LB medium to mid-log phase, and adenylate cyclase activity was induced with the addition of IPTG. Bacterial growth was continued, and the cells were harvested at late-log phase. The culture supernatants and cell lysates were isolated and assayed for adenylate cyclase activity. The adenylate cyclase activities in cell lysates obtained from HtAC-1 and tAC-1 were generally higher than that seen with HAC-1 (Figure 1B) because the truncated *cya*A gene encodes an adenylate cyclase that has an intrinsic activity greater than that encoded by the full-length *cya*A gene. It is not clear, however, why the adenylate cyclase activity in cell lysates

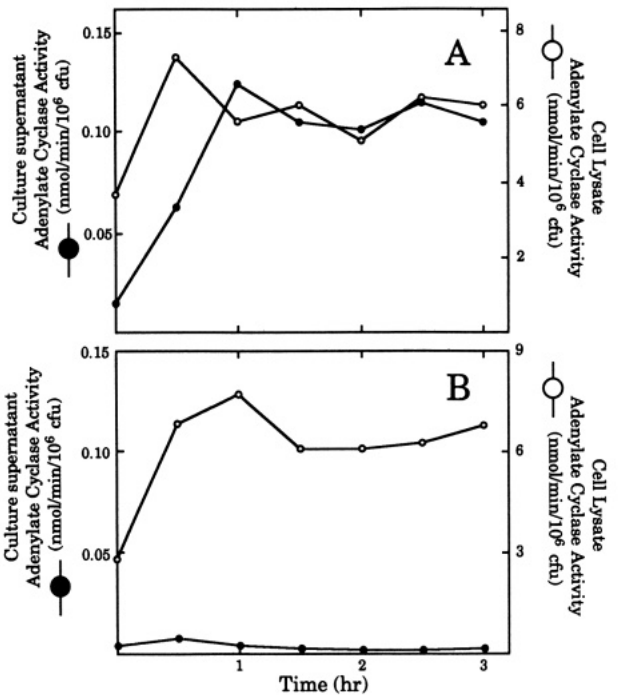

FIGURE 2: Secretion of the *B. pertussis* adenylate cyclase from *E. coli* containing the *hly* operon. Calmodulin-sensitive adenylate cyclase activity was measured in the culture supernatant (●) and cellular extract (○) from the *E. coli* strains HAC-1 (A) and AC-1 (B). HAC-1 is a DH1 derivative that contains the plasmid-encoded adenylate cyclase gene *cya*A (pRM003) and the plasmid-encoded *hly* operon (pSF4000). AC-1 is a DH1 derivative that contains only the plasmid-encoded *cya*A gene (pRM003). Cells were grown to an OD_{650} of 0.4 and adjusted to 1 mM IPTG (time 0), and incubation was continued. Four-milliliter samples were removed at various time points, and culture supernatant and cellular extract were isolated as described under Experimental Procedures. These samples were assayed for calmodulin-sensitive adenylate cyclase activity and expressed as enzyme activity per 10^6 cfu. Each point is the result of duplicate assays, and the standard deviation of each point was less than 2.0% of that value.

obtained from AC-1, the strain harboring the full-length *cya*A without the *hly* operon, was greater than that seen with the same strain containing both the full-length *cya*A gene and *hly* operon. Apparently, expression of proteins encoded by the *hly* operon can affect the activity or stability of *B. pertussis* adenylate cyclase expressed in *E. coli*.

Only HAC-1, the strain of bacteria that harbored the plasmid encoding the full-length *cya*A gene (pRM003) and the plasmid-encoded *hly* operon (pSF4000), secreted adenylate cyclase activity (Figure 1A). HtAC-1, the strain that contained the plasmid with the truncated *cya*A gene and the hemolysin operon, did not secrete adenylate cyclase activity but accumulated adenylate cyclase within the cell (Figure 1B). The truncated gene encodes a protein that is missing its C-terminal 75 amino acids and a putative signal sequence. The strains harboring the full-length *cya*A gene or the truncated *cya*A gene without the *hly* operon did not secrete adenylate cyclase activity.

Localization and Characterization of the B. pertussis Adenylate Cyclase Expressed in E. coli. HAC-1 and AC-1 were grown in LB medium, and the expression of the adenylate cyclase was induced with IPTG as described under Experimental Procedures. Samples were removed at various time points, and the culture supernatant and a soluble bacterial cell extract were isolated from these samples and assayed for adenylate cyclase activity. Enzyme activity increased in both the culture supernatant and cellular extracts of HAC-1 as a function of time after induction (Figure 2A). This is in

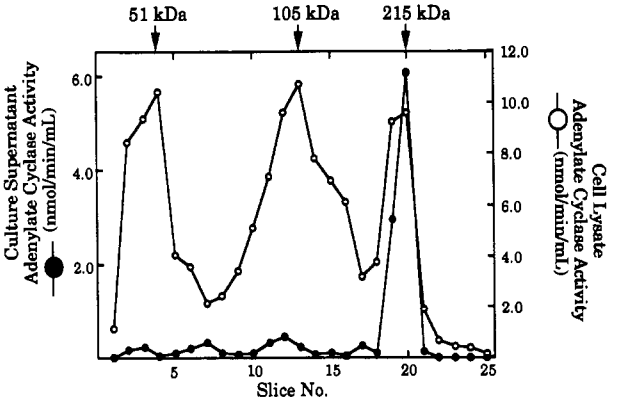

FIGURE 3: Migration profile from SDS–polyacrylamide gel electrophoresis of the culture supernatant and cell-associated forms of the calmodulin-sensitive adenylate cyclase expressed in *E. coli* with the *hly* operon. HAC-1 were grown to an OD_{650} of 0.4 and adjusted to 1 mM IPTG, and incubation was continued for 2 h. The culture supernatant and cellular extract were isolated from 4 mL of media as described under Experimental Procedures. Two hundred microliters of culture supernatant (●) and cellular extract (○) was equilibrated with SDS sample buffer [62.5 mM Tris-HCl, pH 6.8, 1 mM dithiothreitol, 5% (v/v) glycerol, 1% (w/v) SDS, and 0.1% bromophenol blue] and applied to a 7.5% SDS–polyacrylamide gel. Each lane was cut into 0.45-cm slices, and each slice was extracted and assayed for enzyme activity. The culture supernatant and cellular extract applied to the gel contained 2.8 and 60 units (units equal nanomoles per minute) of adenylate cyclase activity, respectively. The apparent molecular masses of the peaks of enzyme activity were determined on the basis of the relative migration of the protein standards myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (92 kDa), bovine serum albumin (64 kDa), and ovalbumin 45 (kDa). Each point is the result of duplicate assays, and the standard deviation of each point was less than 2.5% of that value.

contrast to AC-1 where adenylate cyclase activity was detected only in the cellular extract (Figure 2B). Pretreatment of whole cells or spheroplasts of HAC-1 or AC-1 with trypsin did not alter enzyme activity recovered in cell lysates and demonstrated that cell-associated activity was intracellular. Only 2% of the total adenylate cyclase activity was released into the culture supernatant of HAC-1. This is significantly lower than the percentage of adenylate cyclase activity released from *B. pertussis*. Up to 70% of the total activity is found in the culture supernatant of the Tohama phase I strain of *B. pertussis* while the remainder is cell-associated (Masure & Storm, 1989).

The enzyme activity observed in the culture supernatants from HAC-1 was not due to cell lysis and general release of protein. Plasmid-encoded calmodulin (pVUC-1) was not detected in culture supernatants when expressed in either WAM 582 or DH1 strains of *E. coli* (data not show). Futhermore, the HtAC-1 strain containing the truncated *cya*A gene and the *hly* operon produced significant intracellular adenylate cyclase without release of the enzyme activity into the culture media.

Adenylate cyclase activity recovered from SDS–polyacrylamide gels has been used to purify and characterize different forms of the enzyme (Masure & Storm, 1989). Figure 3 depicts the migration profile of adenylate cyclase activity recovered after SDS–polyacrylamide gel electrophoresis of the culture supernatant and the bacterial cell extract from HAC-1. Only a single peak of enzyme activity with an apparent molecular weight of 215 000 was found in the culture supernatant. Three peaks of enzyme activity with apparent molecular weights of 215 000, 105 000, and 51 000 were found in the bacterial cell extract. The *cya*A gene for the adenylate cyclase encodes a protein with a molecular weight of 177 000. Therefore, only the full-length gene product with an intact C terminus was processed by the hemolysin operon and secreted into the culture supernatant. The smaller forms of enzyme activity found within the bacterial cell were probably proteolytic fragments of the 215-kDa precursor that contain an intact N terminus with catalytic activity but not the C terminus. The variability in the appearance and size of the M_r 105 000 intermediate form of the enzyme from preparation to preparation supports this hypothesis.

Table II: Elevation of cAMP Levels in Mouse Neuroblastoma Cells

enzyme preparation[a]	enzyme applied (nmol min^{-1} $plate^{-1}$)	intracellular cAMP (pmol/mg of protein)[b]
Escherichia coli: peak 1[c]	120	52.0 (±1)
	200	64.7 (±7)
Bordetella pertussis: peak 1[d]	100	5170.0 (±570)
Bordetella pertussis: peak 2[d]	100	66.1 (±1)
no enzyme added	0	42.6 (±11)

[a] Enzyme preparation of adenylate cyclase activity were incubated with mouse neuroblastoma cells as described under Experimental Procedures. Each result is the average of duplicate plates. [b] Intracellular cAMP levels were measured as outlined under Experimental Procedures and standardized to the total amount of cellular protein per plate of cells. [c] Adenylate cyclase was purified from the culture supernatant of *E. coli* (HAC-2) by anion-exchange chromatography. A single peak of enzyme activity eluted at a conductivity of 10 $m\Omega^{-1}$. [d] Invasive (peak 1) and noninvasive (peak 2) preparations of adenylate cyclase were purified from the culture supernatants of *B. pertussis* as described previously (Masure et al., 1988). Peak 1 eluted at a conductivity of 7 $m\Omega^{1}$, and peak 2 eluted at a conductivity of 10 $m\Omega^{-1}$.

The cell-invasive properties of the adenylate cyclase activity purified from *E. coli* culture supernatants were compared with those of an invasive preparation from *B. pertussis*. Up to 200 nmol min^{-1} mL^{-1} enzyme activity secreted from *E. coli* did not have any effect on cAMP levels in mouse neuroblastoma cells (Table II). This is in contrast to the invasive preparation from *B. pertussis* which produced a 10-fold increase in cAMP levels in these cells (Table II). Therefore, the enzyme preparation produced in *E. coli* and secreted by the *hly* operon was not cell-invasive, presumably due to a lack of specific processing of the enzyme in *E. coli* or the requirement of another factor from the *cya* operon required for cell invasion.

Discussion

Previous work from our laboratory has shown that the extracellular calmodulin-sensitive adenylate cyclase produced by *B. pertussis* is synthesized as a large 215-kDa catalytically active precursor (Masure & Storm, 1989). The precursor is processed to a 45-kDa catalytic subunit on the outer surface of the bacterial cell that is released into the culture supernatant. Unlike other secreted bacterial proteins, there was no detectable precursor found in the periplasm [for a review, see Hirst and Welch (1988) or Holland et al. (1989)].

Sequence analysis of the *cya* operon from *B. pertussis*, which contains the gene for the calmodulin-sensitive adenylate cyclase, and the *hly* operon from *E. coli* shows a large degree of similarity in both the structural organization of the genes in the operon and the predicted secondary structure of their gene products (Glaser et al., 1988a,b). Other DNA hybridization studies as well as the cloning and sequencing of the homologous *hly* elements in *Proteus vulgaris*, *Morganella morganii*, and *Pasteurella hemolytica* suggest that the hemolytic determinant is an essential virulence factor associated with these bacteria (Koronakis et al., 1987; Lo et al., 1987; Welch, 1987; Strathdee & Lo, 1989).

Complementation studies have shown that the gene products of *cya*B, *cya*D, and *cya*E are required for the secretion of the adenylate cyclase, the product of the *cya*A gene. By comparison, similar studies with hemolysin have shown that the

gene products of *hly*B and *hly*D are required for the secretion of hemolysin, the gene product of *hly*A (Mackman et al., 1985a,b). Other biochemical and genetic studies with hemolysin suggest a unique molecular mechanism for secretion of this protein from *E. coli*. Unlike other secreted bacterial proteins, hemolysin does not have a cleavable N-terminus signal sequence (Felmlee et al., 1985a). Mackman et al. (1987) have demonstrated that secretion of hemolysin is independent of *sec*A but requires the presence of *Hly*B and *Hly*D for posttranslational export (Mackman et al., 1985a,b, 1986). The last 27 amino acids of the C-terminus region of hemolysin contain a specific signal sequence required for secretion (Nicaud et al., 1986; Mackman et al., 1987; Koronakis et al., 1989). Furthermore, complementation studies with the *hly* determinants from *P. vulgaris* or *M. morganii* and various elements from the *hly* operon from *E. coli* showed that the hemolysins from the *Proteeae* genes were secreted by the gene products from the *E. coli hly* operon (Koronakis et al., 1987).

On the basis of the similarity in the mechanism of secretion of the adenylate cyclase and hemolysin, we hypothesized that the secretion machinery of the *E. coli* hemolysin operon would process the adenylate cyclase from *B. pertussis*. Expression of the *cya*A gene in *E. coli* resulted in the synthesis of the adenylate cyclase with no detectable enzyme activity released into the culture medium. The full-length, 215-kDa, gene product of the *cya*A gene was secreted, however, when expressed in *E. coli* in the presence of the hemolysin operon. A truncated gene missing the final 223 nucleotides from the 3′ end and which codes for the C-terminal 75 amino acids was transcribed, but the gene product was not secreted in the presence or absence of the *hly* operon. In addition, shorter transcripts of 105 and 51 kDa, which contained catalytic activity and probably an intact N terminus, were not secreted. Therefore, sequence-specific information for secretion of the adenylate cyclase by the *hly* operon is probably located in the C terminus of *cya*A.

Even though the full-length, enzymatically active, adenylate cyclase was secreted from *E. coli* when the hemolysin operon was expressed, this form of the enzyme did not elevate intracellular cAMP levels when it was incubated with mouse neuroblastoma cells. This was most likely due either to the lack of another factor required for cell entry or to specific processing of the adenylate cyclase by the gene products of the *cya* operon. In summary, these data indicate that there are enough similarities in the molecular mechanisms for bacterial secretion of hemolysin and *B. pertussis* adenylate cyclase to allow secretion of the adenylate cyclase from *E. coli* when the gene products of the hemolysin operon are expressed.

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Cryoenzymology of Staphylococcal β-Lactamase: Trapping a Serine-70-Linked Acyl-Enzyme†

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ABSTRACT: Various cryosolvents were investigated for their suitability in cryoenzymological experiments with β-lactamase from *Staphylococcus aureus* PC1. On the basis of the minimal effects on the catalytic and structural properties of the enzyme, ternary solvents containing ethylene glycol, methanol, and water were found most suitable. The interaction of β-lactamase with a number of substrates was studied at subzero temperatures. In general, the reaction profiles were similar to those in aqueous solution at above-zero temperatures, with the exception of the slower rates. For cephalosporin substrates, such as PADAC, in which the 3′-substituent may leave to form a more stable form of the acyl-enzyme [Faraci, W., & Pratt, R. (1985) *Biochemistry 24*, 903–910], this intermediate could be readily stabilized at subzero temperatures. At −40 °C the slow rate of deacylation in the reaction with the chromophoric substrate 6β-[(furylacryloyl)amino]penicillanic acid permitted the acyl-enzyme to be stoichiometrically accumulated. This intermediate was then stabilized at low pH with trifluoroacetic acid. Isolation by centrifugal gel filtration, followed by pepsin digestion, gave a penicilloyl-labeled peptide which was isolated by HPLC. Subsequent trypsinolysis of this peptide gave a single labeled peptide, corresponding to the octapeptide surrounding the active-site serine, Ser-70.

β-Lactamases are very efficient enzymes, with turnover numbers of as much as several thousand per second for some substrates. Until recently, investigations of the catalytic mechanism have been hindered by the lack of a high-resolution crystallographic structure. The structure of the enzyme from *Staphylococcus aureus* has now been reported (Herzberg & Moult, 1987). The major role that these enzymes play in resistance to β-lactam antibiotics makes an understanding of their mechanism of action important for the design of mechanism-based antibiotics.

Various investigations of Class A β-lactamases indicate that the catalytic reaction involves the intermediacy of an acyl-enzyme intermediate (Fisher et al., 1980; Anderson & Pratt, 1981, 1983; Cartwright & Fink, 1982; Pratt et al., 1988). Both studies using inhibitors and those in which Ser-70 has been replaced by other residues via site-directed mutagenesis have implicated Ser-70 as an essential nucleophile in catalysis by Class A β-lactamases (Knott-Hunziker et al., 1979; Cohen & Pratt, 1980; Cartwright & Coulson, 1980; Fisher et al., 1981; Clarke et al., 1983; Dalbadie-McFarland et al., 1982; Sigal et al., 1982). Although a number of other potentially essential residues have been identified, either from modification studies or from the structure of the active site, there is little agreement or information regarding the molecular details of the catalytic mechanism.

In the case of β-lactamase from *S. aureus* additional complications may arise from the conformational mobility of the enzyme. For example, a number of penicillins bring about substrate-induced deactivation, a form of reversible inhibition involving conformational effects (Pain & Virden, 1979; Persaud et al., 1986). In addition, intermediate conformational states have been observed under a variety of conditions including low concentrations of denaturant (Robson & Pain, 1976; Creighton & Pain, 1980; Mitchinson & Pain, 1985) and extremes of pH (Carrey & Pain, 1978; R. H. Pain, personal communication).

The short lifetime of enzyme–substrate complexes under normal conditions renders their study difficult. By use of subzero temperatures to slow the catalytic reaction it is possible that intermediates may be accumulated sufficiently to permit some structural information to be obtained (Fink & Cartwright, 1981; Fink & Petsko, 1981). We report here the results from studies aimed at selecting a suitable cryosolvent and demonstrating the presence of an acyl-enzyme involving Ser-70 with a good substrate. There are three main components to this study: the search for a suitable cryosolvent, investigations of the reaction of β-lactamase with various substrates at subzero temperatures, and the trapping and

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