Cloning and characterization of the gene encoding periplasmic 2',3'-cyclic phosphodiesterase of *Yersinia enterocolitica* O:8

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The gene encoding periplasmic 2',3'-cyclic phosphodiesterase in *Yersinia enterocolitica* O:8 (designated *cpdB*), was cloned and expressed in *Escherichia coli*. This enzyme enables *Y. enterocolitica* to grow on 2',3'-cAMP as a sole source of carbon and energy. Sequencing and analysis of a 3 kb *EcoRI* fragment containing the *cpdB* gene revealed an open reading frame of 1179 bp, corresponding to a protein with a molecular mass of 71 kDa. The first 25 amino acid residues show features of a typical prokaryotic signal sequence. The predicted molecular mass of the mature peptide is therefore in agreement with the molecular mass estimated by SDS gel electrophoresis (68 kDa). The putative *cpdB* promoter region contains two possible –10 and –35 regions. Furthermore, the 5' untranslated region contains sequences with significant homology to the cyclic AMP–cyclic AMP receptor protein binding site and the σ^{28} consensus. This region is interrupted by an enterobacterial repetitive intergenic consensus (ERIC) sequence. Deletion of the ERIC element from the *cpdB* promoter region had no effect on *cpdB* expression. In the 3' untranslated region, a possible rho-independent transcriptional terminator was identified. The deduced amino acid sequence of the *Y. enterocolitica* CpdB protein shows 76% identity with CpdB of *Salmonella typhimurium* and *E. coli*. CpdB of *Y. enterocolitica* is exported to the periplasmic space. An isogenic *Y. enterocolitica* *cpdB* mutant strain, constructed by allelic exchange, was no longer able to grow on 2',3'-cAMP as sole source of carbon and energy. The CpdB mutant showed no significant change in virulence in an oral and intravenous mouse infection model.

Keywords: *cpdB* gene, 2',3'-cAMP, ERIC, cAMP–CRP-binding site

INTRODUCTION

Bacterial periplasmic 2',3'-cyclic phosphodiesterases (CpdB) are bifunctional enzymes which possess 2',3'-cyclic phosphodiesterase as well as 3'-nucleotidase activity. They catalyse the hydrolysis of 2',3'-cyclic phosphates of adenosine, guanosine, cytosine and uridine according to the following reaction: 2',3'-cyclic

AMP $\rightarrow$ 3'-AMP $\rightarrow$ adenosine + phosphate. Neither nucleotide sugars nor 3',5'-cyclic nucleotides are hydrolysed (Liu & Beacham, 1990; Neu, 1968a). Furthermore, CpdB is able to hydrolyse the chromogenic substrates *p*-nitrophenyl phosphate (PNPP), bis(PNPP) and *p*-nitrophenyl phosphorylcholine (NPPC). Bacterial 2',3'-cyclic phosphodiesterases are related to 5'-nucleotidases, which are multifunctional enzymes that hydrolyse nucleoside 5'-tri-, 5'-di- and 5'-monophosphates (Zimmermann, 1992). 2',3'-Cyclic phosphodiesterases are intermediates in the hydrolysis of RNA by ribonuclease I, which is also a periplasmic enzyme and is unable to generate transportable substrates (Abrell, 1971). These findings have led a number of authors to suggest a

Abbreviations: cAMP–CRP, cyclic AMP–cyclic AMP receptor protein; ERIC sequence, enterobacterial repetitive intergenic consensus sequence; NPPC, *p*-nitrophenyl phosphorylcholine; PNPP, *p*-nitrophenyl phosphate.

The GenBank accession number for the *cpdB* gene fragment of *Yersinia enterocolitica* O:8 strain WA-314 reported in this paper is X85742.

possible scavenging function for these enzymes, hydrolysing non-transportable nucleotides produced by RNase I to nucleosides that can easily enter the cell and which could be used subsequently as a source of carbon and energy (Liu & Beecham, 1990; Neu, 1968a, b). 2',3'-Cyclic phosphodiesterase activity has been described for most members of the *Enterobacteriaceae* as well as for *Haemophilus influenzae* (Anderson *et al.*, 1985; Neu, 1968b) and *Vibrio* spp. (Dunlap & Callahan, 1993; Unemoto *et al.*, 1969). Non-microbial 2',3'-cyclic phosphodiesterases have been reported from a wide array of vertebrate sources. In particular, the enzymes from the brain and the retina are expressed at high levels, but their physiological significance is poorly understood (Vogel & Thompson, 1988). The pH optimum of enterobacterial cyclic phosphodiesterases ranges from 7.2 to 7.8 and metal stimulation is greatest with Co^{2+} . Protein inhibitors of CpdB have not been found, but ribonucleosides were shown to inhibit CpdB activity (Neu, 1968b). The genes encoding the 2',3'-cyclic phosphodiesterase of *Escherichia coli* and *Salmonella typhimurium* have been cloned and characterized (Liu *et al.*, 1986; Liu & Beacham, 1990). Both genes were shown to be moderately regulated by carbon-source availability through the cyclic AMP–cyclic AMP receptor protein (cAMP–CRP) complex. Expression of CpdB increased when bacteria were grown on poor carbon sources, but not when they were grown on poor nitrogen or phosphorus sources (Kier *et al.*, 1977; Liu *et al.*, 1986). 2',3'-Cyclic phosphodiesterases, like other bacterial phosphatases (alkaline phosphatase, 5'-nucleotidase, acid hexose phosphatase, non-specific acid phosphatase), are believed to be periplasmic in location, which is supported by the release of these enzymes by osmotic shock and the hydrolysis of phosphate esters and diester substrates by intact cells (Neu & Chou, 1967). It should be noted that a different periplasmic bacterial cyclic phosphodiesterase, the 3',5'-cyclic nucleotide phosphodiesterase (CpdP), has been cloned and characterized from *Vibrio fischeri* (Dunlap & Callahan, 1993) and *Yersinia enterocolitica* (Young & Miller, 1997). 3',5'-Cyclic AMP phosphodiesterase of *Y. enterocolitica* was identified as a host-responsive element in an oral mouse infection model and is believed to enhance the survival of yersiniae within the host's Peyer's patches. In this report, we describe the cloning and sequencing of *cpdB* from *Y. enterocolitica* O:8 and the construction of a *cpdB* mutant to elucidate the contribution of *cpdB* to pathogenicity in a mouse model. Furthermore, the role of the enterobacterial repetitive intergenic consensus (ERIC) element in the regulation of CpdB was analysed. This is the first report on CpdB production by *Yersinia* spp.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* HB101 was used for subcloning *cpdB*, and S17-1 λ pir was used as a host for the suicide vector, pGPCAT. Bacteria were cultured aerobically in Luria–Bertani (LB) broth or on LB agar plates (Difco) at 27 °C (*Yersinia*) or 37 °C (*E. coli*). M9 medium

(Miller, 1972) was supplemented with appropriate carbon and energy sources (glucose, 2',3'-cAMP) at 5 mM. Antibiotics were used at the following concentrations ($\mu\text{g ml}^{-1}$): ampicillin, 100; kanamycin, 25; nalidixic acid, 60; chloramphenicol, 20; tetracycline, 20; carbenicillin, 250.

Nucleic acid manipulations. Plasmid DNA preparations were isolated with Qiagen kits according to the manufacturer's recommendations. Conjugations between bacterial strains were performed overnight on blood agar plates as described previously (Heesemann & Laufs, 1983). Restriction enzyme digests, recovery of DNA fragments from agarose gels by using DEAE membrane (Schleicher & Schuell), ligations, transformations and Southern blot hybridizations were performed as described by Ausubel *et al.* (1989). Enzymes, deoxynucleoside triphosphates and *Taq* polymerase were purchased from Roche Molecular Biochemicals. The digoxigenin-dUTP labelling and detection kit (Roche Molecular Biochemicals) was used for DNA probes in hybridization experiments. Oligonucleotides were synthesized by Metabion and Roth. The construction of the gene bank from *Y. enterocolitica* WA-C in the cosmid vector pLAFR2 is described elsewhere (Rakin *et al.*, 1994).

The *cpdB* gene was isolated from the gene bank by screening with NPPC. Restriction enzyme digests of cosmids with *Sau*3AI and religation in pACYC177 restricted with *Bam*HI resulted in pKT2. After digestion with *Eco*RI and religation with pACYC177, a 3 kb *Eco*RI fragment, which was still positive in the NPPC assay, was isolated (pKT2.1). For sequencing, this 3 kb *Eco*RI fragment was subcloned in pBluescript KS(–), resulting in pKT3. The nucleotide sequence of the 3 kb *Eco*RI fragment was determined using the *Taq* DyeDideoxy terminator method with a 373A DNA Sequencer (Applied Biosystems). Nested deletions were constructed using the Double-stranded Nested Deletions Kit (Pharmacia). Universal primers used to sequence the *cpdB* gene were as follows: forward, 5'-GTAAAACGACGGC-CAGT-3'; reverse, 5'-CAGGAAACAGCTATGAC-3'. Non-overlapping regions were sequenced using specific oligonucleotides. Sequences were analysed and aligned with the HIBIO Macintosh DNASIS program (Hitachi Software Engineering) and with the Genetics Computer Group sequence-analysis software package (University of Wisconsin, Madison, WI, USA).

A *cpdB* mutant of *Y. enterocolitica* was constructed by introducing a 1.2 kb Km-GenBlock derived from pUC4K (Pharmacia LKB) into the *Bal*I site of pKT3, resulting in pKT3.K. The *Bal*I site is located inside the *cpdB* gene, 779 bp downstream of the start codon. The *cpdB*::Km gene fragment was transferred to the suicide vector pGPCAT (Roggenkamp *et al.*, 1995) by using the *Sal*I and *Sac*I restriction sites. The resulting plasmid, pKT4.K, was mobilized into WA-314. Exconjugants resistant to nalidixic acid and kanamycin but sensitive to chloramphenicol were further characterized. The allelic exchange [disruption of *cpdB* by the insertion of a kanamycin-resistance (Km^{R}) cassette] resulting in the WA-314 *cpdB* mutant (WA-314_{*cpdB*}) was confirmed by Southern hybridization with digoxigenin (DIG)-labelled PCR probes as specified by Boehringer Mannheim Biochemica.

To study *cpdB* gene expression, translational fusions between *cpdB* promoter and luciferase were constructed by using PCR cloning procedures. Primers were designed not to produce frameshifts. The *cpdB* promoter region was amplified using the forward primer 5'-CCCAAGCTTCTTCTCAATAAAAA-TAAGGGAA-3', preceded by a *Hind*III restriction site, and

Table 1. Strains of *Yersinia* and *E. coli* and plasmids used in this study

Strain or plasmid	Description*	Reference or source
<i>Yersinia</i>		
WA-314	<i>Y. enterocolitica</i> serotype O:8; clinical isolate; pYVO8 ⁺	Heesemann <i>et al.</i> (1983)
WA-C	Plasmidless derivative of WA-314	Heesemann <i>et al.</i> (1983)
WA-C::pKT7	WA-C with chromosomal integration of pKT7	This study
WA-C::pKT8	WA-C with chromosomal integration of pKT8	This study
WA-314 _{cpdB}	<i>cpdB</i> mutant of WA-314; Km ^R	This study
WA-314 _{cpdB} (<i>cpdB</i>)	WA-314 _{cpdB} complemented with pKT3	This study
Y-108-C	<i>Y. enterocolitica</i> clinical isolate, serogroup O:3 pYV ⁻	Heesemann (1987)
Y-96-C	<i>Y. enterocolitica</i> clinical isolate, serogroup O:9 pYV ⁻	Heesemann (1987)
NF-O	<i>Y. enterocolitica</i> clinical isolate, serogroup O:5	Ratnam <i>et al.</i> (1982)
Y-P-I	<i>Y. pseudotuberculosis</i> clinical isolate, serogroup O:1	Heesemann (1987)
Y-P-III	<i>Y. pseudotuberculosis</i> clinical isolate, serogroup O:3	Heesemann (1987)
Y-P-I(<i>cpdB</i>)	Y-P-I complemented with pKT3	This study
Y-P-346	<i>Y. pseudotuberculosis</i> clinical isolate, serogroup O:3	Heesemann (1987)
KIM	<i>Y. pestis</i> human isolate; Lcr ⁻ Pgm ⁺	R. R. Brubaker
KUMA	<i>Y. pestis</i> human isolate; Lcr ⁻ Pgm ⁺	R. R. Brubaker
YOKOHAMA	<i>Y. pestis</i> human isolate; Lcr ⁻ Pgm ⁺	R. R. Brubaker
<i>Y. kristensenii</i>	Animal isolate	Heesemann (1987)
<i>Y. intermedia</i>	Environmental isolate	Heesemann (1987)
<i>E. coli</i>		
HB101	(<i>gpt-proA</i>)62 <i>leuB6 thi-1 lacY1 hsdS_B20 recA rps-20(Str^R) ara-14 galK2</i>	Boyer <i>et al.</i> (1969)
DH5 α	<i>endA1 hsdR17(r_k⁻ m_k⁺) supE44 thi-1 recA1 gyrA(Nal^R) relA1</i> $\Delta(lacZYA-argF)_{U169}$ (ϕ 80 <i>lacZ</i> Δ M15)	Hanahan (1983)
S17-1 λ pir	<i>pir⁺ tra⁺</i> , Km ^R	Simon <i>et al.</i> (1988)
Plasmid		
pLAFR2	21.6 kb; Mob ⁺ , Tet ^R	Friedmann <i>et al.</i> (1982)
pKS	2.4 kb; Amp ^R , <i>lacZ</i> ⁺	Stratagene
pT7-5	2.21 kb; pMB1, Ap ^R	Tabor & Richardson (1985)
pGP1-2	7.14 kb; gene I cI857	Tabor & Richardson (1985)
pGPCAT	Suicide vector; R6K replicon; Cm ^R Mob ⁺	Roggenkamp <i>et al.</i> (1995)
pUC4K	Kanamycin cassette (Km ^R)	Pharmacia-LKB
pKAS 32	pGP704, <i>rpsL</i>	Skorupski & Taylor (1996)
pCJYE138-L	<i>Sall-HindIII yopE138-luc</i> fragment in pACYC184	Jacobi <i>et al.</i> (1998)
pACYC177	Low-copy vector; Km ^R Amp ^R	N. E. BioLabs
pACYC184	Low-copy vector; Cm ^R Tc ^R	N. E. BioLabs
pKT1	pLAFR2 with 20 kb insert encoding <i>cpdB</i> from WA-C	This study
pKT2	pACYC177 with 12 kb insert encoding <i>cpdB</i> from WA-C	This study
pKT2.1	pACYC177 with 3 kb <i>EcoRI</i> insert encoding <i>cpdB</i> from WA-C	This study
pKT3	pKS with 3 kb <i>EcoRI</i> fragment from pKT2.1	This study
pKT4.1	3 kb <i>EcoRI</i> fragment from pKT 2.1 in pT7-6, 5'-3' orientation	This study
pKT4.2	3 kb <i>EcoRI</i> fragment from pKT 2.1 in pT7-6, 3'-5' orientation	This study
pKT3.K	pKT3 with <i>cpdB</i> inactivated by a Km-GenBlock in the <i>BalI</i> site of <i>cpdB</i> (<i>cpdB::Km</i>)	This study
pKT4.K	pGPCAT with <i>cpdB::Km</i>	This study
pKT5	pCJYE138-L with YopE fragment replaced by <i>BamHI HindIII cpdB</i> PCR fragment	This study
pKT6	pKT5 with deletion of bp 581-708 (ERIC) of <i>cpdB</i> promoter	This study
pKT7	<i>cpdB</i> PCR fragment from pKT5 in pKAS 32	This study
pKT8	<i>cpdB</i> PCR fragment with ERIC deletion from pKT6 in pKAS 32	This study

the reverse primer 5'-CGCGGATCCAGTACTCGCAAA-TC-3', followed by a *BamHI* restriction site. Plasmid pCJYE138-L was digested with *BamHI* and *HindIII* and ligated with the *HindIII*- and *BamHI*-restricted PCR fragment, resulting in pKT5.

An ERIC deletion in the *cpdB* promoter region was generated by amplification of pKT5 with primers 5'-ATTAAATTT-ACATATTCTTTTGGCGATACAGGTCTGA-3' and 5'-GGT-CGTCATAACAAAGTGTGAAGTTTGGCAGAAAAT-3', which have a corresponding gap between their 5'-ends. This

was accomplished using TaKaRa LA *Taq* polymerase (TAKARA Biotechnology) and a GenAmp system 2400 cyclor (Perkin-Elmer) with the following thermal profile: denaturation at 94 °C for 15 min, 30 cycles of 98 °C for 20 s and 68 °C for 15 min, followed by 72 °C for 10 min. The amplified DNA fragment was treated with T4 DNA polymerase, to generate blunt-ended DNA, self-ligated and transformed into *E. coli* DH5 α and *Y. enterocolitica* WA-314. Sequence analysis of the resulting pKT6 confirmed the deletion of bases 581–708. To integrate the reporter system into the chromosome, the *cpdB*–luciferase constructs in pKT5 and pKT6 were subcloned into the suicide vector pKAS 32, using the *Xba*I and *Sal*I restriction sites after partial digestion (due to the *Xba*I restriction site in the luciferase gene). The resulting plasmids (pKT7, pKT8) were transferred, by conjugation, into WA-C. PCR analysis with primer 5'-CCATCGATTAGCGCTGCC-AGTGCT-3', lying upstream of the cloned *cpdB* fragment, and a reverse primer within the luciferase gene (5'-AGTAT-TCCGCGTACGTGA-3') confirmed the expected integration resulting from homologous recombination via the *cpdB* promoter region. Primers used to screen for the presence of the *cpdB* gene and the ERIC element in other *Yersinia* spp. were 5'-ATCAGGTCGCCATTATCTAG-3' and 5'-TTCTGCCA-ACTTCACACTT-3'.

Quantification of *cpdB*–*luc* gene expression. To study *cpdB*–*luc* gene expression, a luciferase reporter gene assay was performed as described by the manufacturer (Roche Molecular Biochemicals). Bacteria were grown overnight at 28 °C, diluted 1:40 and then grown to exponential phase. The amount of bacteria was standardized by measuring the OD₆₀₀ and plating the bacteria. The yersiniae were lysed and centrifuged, and the supernatant was transferred to a microtitre plate (Dynatech). Luciferin substrate was added and the emitted photons were counted for 10 min by a CCD camera. Activity was measured in a darkbox with a microtitre plate chemiluminometer (CCD camera C2400-77; Hamamatsu Photonics).

CpdB activity assays. CpdB activity was determined by measuring absorbance at 410 nm after incubation with 5 mM bis(PNPP) or 20 mM NPPC for 20 min at 37 °C, in substrate buffer: (5 mM CoCl₂, 1 mM MgCl₂, 50 mM Tris/maleate, pH 7.8) or (250 mM Tris/HCl, 1 mM Zn²⁺, 10 mM NaF, 45% sorbitol), respectively.

Overexpression of *cpdB*. *cpdB* was overexpressed in *E. coli* using the temperature-sensitive T7 RNA polymerase/promoter system of Tabor & Richardson (Ausubel *et al.*, 1989). The 3 kb *Eco*RI insert from pKT2.1 was cloned into pT7-6, restricted with *Eco*RI, in 3'–5' and 5'–3' orientation, resulting in pKT4.1 and pKT4.2, respectively. These plasmids were transformed into *E. coli* HB101 harbouring pGP1-2. T7 RNA polymerase was induced by incubating the bacteria at 42 °C and whole-cell lysates were analysed by SDS-PAGE.

Isolation of subcellular fractions. Bacterial cells were fractionated as described by Ölschläger & Braun (1987). The sediment from 10 ml bacterial culture was resuspended in 1.3 ml 3 mM Tris/HCl, 30 mM sucrose/0.03 mM EDTA solution, pH 8.0, containing 10 µg lysosyme. The suspension was frozen and thawed twice. After 45 min at room temperature, it was centrifuged. The supernatant contained periplasmic and cytoplasmic proteins. The sediment was resuspended in 1.2 ml 20 mM MgCl₂ containing 5 µg bovine DNase. After 1 h incubation, the suspension was centrifuged at 30000 g for 60 min. Cytoplasmic proteins present in the supernatant were precipitated with ethanol. Membrane proteins were located in the sediment. Periplasmic proteins were released using the

chloroform-shock method described by Ferro-Luzzi Ames *et al.* (1984).

Mouse virulence tests. Virulence was tested in the intravenous and orogastric mouse infection models as described previously (Roggenkamp *et al.*, 1995). For the intravenous and oral challenge, BALB/c mice (6- to 8-week-old females; Charles River WIGA) were infected with 4×10^4 (40 times the minimal lethal dose) (Vogel & Thompson, 1988) and 2×10^8 bacteria, respectively. Mice were killed 2 and 4 d after the intravenous challenge and 5 d after the oral challenge. The number of bacteria in each organ was determined by plating serial dilutions of homogenized tissue.

RESULTS

Cloning and sequencing of the *Y. enterocolitica cpdB* gene

The gene encoding CpdB of *Y. enterocolitica* O:8 strain WA-C was isolated by screening a cosmid library constructed in pLAFR2 for the ability to hydrolyse NPPC. Positive clones were identified by the liberation of yellow *p*-nitrophenolate from NPPC-containing substrate agar. To identify coding sequences on a 20 kb NPPC-positive cosmid, partial *Sau*3AI digests were subcloned in pACYC177 restricted with *Bam*HI. After transformation into HB101, a 12 kb clone that still retained NPPC activity was isolated. This clone was digested with *Eco*RI, religated and transformed into HB101. The smallest plasmid isolated that still retained activity in the NPPC assay contained a 3 kb *Eco*RI fragment. This fragment was subcloned into pKS, resulting in pKT3. A library of nested deletions was created, which allowed sequencing of the entire 3 kb fragment with universal primers. Two gaps were sequenced using specific oligonucleotides. The DNA sequence of pKT3 revealed an open reading frame starting with the ATG in position 779 and ending with the TAA in position 2735 (GenBank accession no. X85742).

Sequence comparison

We compared the deduced amino acid sequence of the open reading frame on pKT3 with sequences in the EMBL and SWISS-PROT protein-sequence databases. Alignment by standard computerized methods revealed identities of 76% with *E. coli* (accession no. P08331) and *S. typhimurium* (accession no. P26265) CpdB (Liu *et al.*, 1986; Liu & Beacham, 1990), 60% with *H. influenzae* CpdB (accession no. P44764), 42% with the *Bacillus subtilis* CpdB homologue (accession no. BAA08981.1) (Yamamoto *et al.*, 1996), 33% with the putative nucleotidase of *Streptomyces coelicolor* (accession no. CAB52071.1), 26% with *Helicobacter pylori* CpdB (accession no. AAD05687), 25% with *Clostridium perfringens* CpdB (accession no. BAA81646.1), 57% with a fragment of the *Aeromonas hydrophila* CpdB homologue (accession no. s57941), as well as slightly more than 20% with 5'-nucleotidases and UDP-sugar hydrolases from a wide spectrum of bacterial as well as vertebrate sources. A search in the Sanger Centre BLAST

server revealed a gene of *Yersinia pestis* that is 81% identical to the *cpdB* gene of *Y. enterocolitica*. No other 2',3'-cyclic phosphodiesterases (human, eukaryotic) had significant similarity to the *Y. enterocolitica* sequence, as determined by the BLAST program. Bacterial 2',3'-cyclic phosphodiesterases (EC 3.1.4.16), 5'-nucleotidases (EC 3.1.3.5) and mosquito apyrase (EC 3.6.1.5) belong to a group of related proteins that have several highly conserved regions in common. Two of these conserved regions, which could have considerable functional significance, are located in the N-terminal ends of these enzymes. These signature patterns (LIVM)-x-(LIVM)-(LIVM)-(HEA)-(TI)-x-D-x-H-(GSA)-x-(LIVMF) and (FYP)-x-x-x-x-(LIVM)-GNHEF-(DN) (Zimmermann, 1992), the second of which contains a perfectly conserved pentapeptide, were also found in CpdB of *Y. enterocolitica*.

Analysis of nucleotide sequence and leader peptide

Analysis of the promoter region revealed a ribosome-binding site (GGAGA) 6 bp upstream of the start codon in position 779. Furthermore, the nucleotide sequence revealed two putative -35 (position 712 TTTACA, 725 TTGCGA) and -10 regions (737 TCGAAT, 749 CTTAAT). In position 2767, a palindromic sequence, which could form a stem-loop structure consisting of a 10-base stem and a 5-base loop was identified downstream of the potential stop codon (TAA in position 2735). This structure could act as a rho-factor-independent transcriptional terminator. The free energy calculated by the method of Tinoco is $-21.5 \text{ kcal mol}^{-1}$ ($-89.9 \text{ kJ mol}^{-1}$) (Tinoco *et al.*, 1973), which is in the range of a stable stem. Another characteristic of rho-independent terminators is the TCGT consensus sequence (Brendel & Trifono, 1984), which is located downstream of the terminator. Three sequences starting in positions 2791, 2800 and 2865 have similarities to this sequence.

The deduced amino acid sequence beginning with the ATG in position 779 shows an N-terminal region of 24 amino acids (MFKRPLTSLASLIALTTSTAQAA) with features of a typical prokaryotic signal sequence (Pugsley, 1993). The N-terminal region contains three positively charged amino acids (lysine, arginine and proline) followed by a hydrophobic span that is rich in alanine and leucine. Furthermore, the two alanine residues in positions 24 and 25 represent the cleavage site of a signal peptidase (Ratnam *et al.*, 1982). The most likely initiation and termination codons in positions 779 and 2735 encode a protein with a molecular mass of 71958 kDa, which includes the signal sequence with a molecular mass of 2548 kDa. This is in agreement with a molecular mass of 68 kDa determined for the mature peptide by using SDS-PAGE (Fig. 1). A second open reading frame was identified upstream of the CRP-binding site, from position 288 to the 5'-end of the sequenced fragment, which shows high homology to the *E. coli* *cysQ* gene, which is needed for cysteine synthesis (Neuwald *et al.*, 1992). A methionine codon was found in position 286 and a Shine-Dalgarno (SD) sequence in

Fig. 1. Coomassie-blue-stained SDS-polyacrylamide gel with cell extracts from *E. coli* HB101 carrying different plasmids induced in the T7 RNA polymerase/promoter system. Lanes: 1, pT7-6 with a 3 kb *EcoRI* insert harbouring the *cpdB* gene in the 3'-5' orientation; 2, pT7-6 with a 3 kb *EcoRI* insert harbouring the *cpdB* gene in the 5'-3' orientation; 3, pT7-6 without the insert.

position 295, indicating that this gene is transcribed in the opposite direction with respect to the *cpdB* gene.

Sequences from position 513 to 533 AAC-TGTGAGCATCTTGCGCTT have good homology to the consensus sequence AANTGTGANNTA-NNTCACATT of the CRP-cAMP complex including the highly conserved TGTGA motif (Kolb *et al.*, 1993) (Fig. 2). The CRP-cAMP-binding site is also present in the promoters of *cpdB* genes from *E. coli* and *S. typhimurium* and is responsible for modulation of gene expression according to carbohydrate-source availability (Liu & Beacham, 1990). Sequences from position 708 to position 734 TAAA(N₁₅)GCGATAC revealed good agreement with the σ^{28} consensus sequence TAAA(N₁₅)GCCGATAA (conserved residues are indicated by underlining). Sequences with similarity to σ^{28} were not found in the promoter regions of *cpdB* from *E. coli* and *S. typhimurium*. Sigma factors direct late transcription of flagellin, chemotaxis and motility genes (Iriarte *et al.*, 1995). Downstream of the CRP-cAMP complex in *Y. enterocolitica* *cpdB*, we identified a 126 bp ERIC sequence (also known as an IRU) (Hulton *et al.*, 1991). This ERIC sequence is 88% identical to the consensus sequence. Interestingly, in places where the *Y. enterocolitica* ERIC sequence differs from the consensus sequence, a compensatory base change is seen in the complementary arm of the stem-loop structure (Fig. 2). ERIC sequences are not present in the promoter regions of *cpdB* genes of *E. coli* and *S. typhimurium*. Sequencing analysis of PCR products showed that the 5' untranslated regions of *cpdB* from *Y. enterocolitica* O:3 and *Y. enterocolitica* O:9 also contain ERIC sequences, whereas *Y. pseudotuberculosis*, *Y. pestis*, *Yersinia kristensenii*, *Yersinia intermedia*, as well as the non-pathogenic *Y. enterocolitica* NF-O, do not harbour ERIC sequences

Table 3. CpdB activity (arbitrary units) of different *Yersinia* spp. determined by hydrolysis of bis(PNPP) in whole cells under different growth conditions

Results are means $\pm$ SD from three independent experiments.

Strain	Growth conditions			
	LB (27 °C)	LB (27 °C) + glucose	LB (27 °C) + cAMP	LB (37 °C)
<i>Y. pestis</i> Kim	1.79 $\pm$ 0.38			
WA-314	3.98 $\pm$ 0.42	1.76 $\pm$ 0.21	3.12 $\pm$ 0.52	1.32 $\pm$ 0.38
WA-314 _{cpdB}	0.18 $\pm$ 0.10			
WA-314 _{cpdB} (<i>cpdB</i>)	1.81 $\pm$ 0.23			
Y-P-I	0.28 $\pm$ 0.12			
Y-P-I(<i>cpdB</i>)	1.24 $\pm$ 0.28			

Table 4. Intensity of luminescence (arbitrary units) of WA-C harbouring chromosomal *cpdB-luc* fusions containing the ERIC element (WA-C::pKT7) or lacking the ERIC element (WA-C::pKT8)

Results are means $\pm$ SD from three independent experiments.

Growth medium	WA-C::pKT7	WA-C::pKT8
LB (mid-exponential; 27 °C)	40.6 $\pm$ 5.6	41.8 $\pm$ 3.8
LB (late exponential; 27 °C)	33.8 $\pm$ 3.7	28.6 $\pm$ 2.1
LB (37 °C)	11.3 $\pm$ 3.8	14.0 $\pm$ 0.8
LB + glucose (10 mM; 27 °C)	15.1 $\pm$ 2.4	12.8 $\pm$ 1.2
LB + 2',3'-cAMP (10 mM; 27 °C)	37.8 $\pm$ 3.2	34.2 $\pm$ 2.2

merase/promoter system were fractionated into periplasmic, cytoplasmic, inner-membrane and outer-membrane proteins. Cellular fractions of bacteria induced at 42 °C were compared with non-induced fractions by SDS-PAGE. Fig. 3 shows the periplasmic location of the product of the induced *cpdB* gene. CpdB was not detected in cell-free supernatant. Enzyme activity was detected in all three pathogenic *Yersinia* species, i.e. *Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis*, by the hydrolysis of bis(PNPP) measured in whole cells (Table 3). Activity was highest for *Y. enterocolitica* WA-314 and lowest for *Y. pseudotuberculosis*. This assay, however, may not accurately reflect CpdB activity, since other enzymes, such as the *Y. pestis* murine toxin (phospholipase D), have also been reported to hydrolyse PNPP (Rudolph *et al.*, 1999).

Regulation of CpdB

To study the promoter activity, especially the influence of the ERIC element on cyclic phosphodiesterase expression, CpdB-luciferase protein fusions were con-

structed. The promoter region (296 bp) and the first 32 amino acids of *cpdB* were amplified by PCR. After restriction enzyme digests with *Bam*HI and *Hind*III, this fragment was ligated into the low-copy plasmid pACYC184 containing the reporter gene luciferase (*luc*) of the firefly *Photinus pyralis* (Jacobi *et al.*, 1998). To generate an ERIC deletion, this plasmid, pKT5, was amplified with primers that have a corresponding gap (containing the ERIC element) between their 5'-ends. This fragment was self-ligated after the generation of blunt ends with T4 DNA polymerase. These plasmids were transformed into *Y. enterocolitica* WA-314 and the chemiluminescence of *cpdB-luc* fusions was measured with a CCD camera, after the addition of luciferin. To exclude copy-number effects, these constructs were integrated into the chromosome of WA-C. *cpdB-luc* fusions lacking the ERIC element (bases 581–708) did not show a significant difference in luminescence from fusions with the wild-type promoter under different growth conditions (Table 4). The luminescence of *cpdB-luc* fusions was higher when bacteria were grown at 27 °C than when growth was at 37 °C. Furthermore, strains grown with added glucose showed lower luminescence than those without added glucose, which suggests that *cpdB* is catabolite-repressed and weakly regulated by the cAMP–CRP complex. Expression was highest during mid-exponential growth (Table 4). These results were verified by measuring CpdB activity directly from bacteria by using the coloured substrate bis(PNPP) (Table 3).

Growth of *Y. enterocolitica* on 2',3'-cAMP as sole source of carbon and energy

While studying a possible function of CpdB in metabolizing nucleotides from the environment, we discovered that *Y. enterocolitica* and *Y. pestis*, as well as the non-pathogenic strains *Y. kristensenii* and *Y. intermedia*, were able to grow readily on M9 minimal agar supplemented with 2',3'-cAMP as the sole carbon and energy source. (Table 2). This prompted us to determine whether this unusual ability was attributable to *cpdB*

Fig. 4. Growth of *Yersinia* spp. in M9 medium containing cAMP (5 mM) as the sole source of carbon and energy. ■, *Y. enterocolitica* WA-314; ◆, *Yersinia pestis* KIM; □, *Yersinia pseudotuberculosis* I(cpdB); △, *Y. enterocolitica* WA-314_{cpdB}; ▲, *Yersinia pseudotuberculosis* I; +, WA-314_{cpdB}.

expression. In accord with this assumption, the *Y. enterocolitica cpdB* mutant strain was no longer able to grow on M9 medium containing 2',3'-cAMP as the sole source of carbon and energy. Growth of *Yersinia* spp. on M9 minimal medium supplemented with 2',3'-cAMP was not due to carry-over nutrients from rich media or from components of the minimal agar, since yersiniae were unable to grow on M9 medium not supplemented with 2',3'-cAMP. We were able to partially complement the growth phenotype of WA-314_{cpdB} with pKT3 encoding the wild-type *cpdB* gene. Furthermore, transformation of Y-P-I, which does not show the growth phenotype, with pKT3 enabled this strain to grow in M9 medium supplemented with 2',3'-cAMP as the sole source of carbon and energy (Fig. 4).

Virulence of WA-314_{cpdB} for mice

To determine whether CpdB contributes to the virulence of *Y. enterocolitica* in a mouse model, the *Y. enterocolitica cpdB* mutant strain was studied in an orogastric and intravenous mouse infection model. The progress of infection was monitored by determining the number of surviving bacteria in the spleen and the liver after intravenous infection, and in Peyer's patches, mesenteric lymph nodes and the spleen after orogastric infection. Two groups of four BALB/c mice were infected orally with 2×10^8 bacteria of strain WA-314 or WA-314_{cpdB}, respectively. The results are summarized in Table 5. On day 5, the numbers of reisolated bacteria in Peyer's patches, the spleen and mesenteric lymph nodes were not significantly lower for the *cpdB* mutant than for the wild-type strain. The course of infection was progressive, with dissemination of bacteria to lymphatic organs on day 5 (as is the case for the wild-type strain). For the intravenous infection route, we determined bacterial loads in the spleen and the liver 2 and 4 d after infection of two groups of three of BALB/c mice with 4×10^4 bacteria of strain WA-314 and strain WA-314_{cpdB}, respectively. In terms of the number of bacteria isolated, there was no significant difference between WA-314_{cpdB} and the wild-type strain. The mutant strain was still able to rapidly colonize the host and multiply in the liver and the spleen, as is the case for the wild-type strain.

DISCUSSION

Bacterial 2',3'-cyclic phosphodiesterases have been extensively characterized in the *Enterobacteriaceae* since their discovery by Anraku (1964). Their physiological significance, however, remains enigmatic. CpdB probably has a scavenging or fitness function, enabling bacteria to utilize exogenously supplied 2',3'-cyclic nucleotides produced by ribonucleases (Cannistraro & Kennell, 1991) as a sole source of carbon and energy. Interestingly, Neu (1968b) reported that several bacterial strains that lacked ribonuclease I (including *Pseudo-*

Table 5. Number of bacteria per organ in BALB/c mice 2 and 4 d after intravenous infection with 4×10^4 bacteria and 5 d after orogastric infection with 2×10^8 bacteria

Results are means from three mice (for intravenous infection) or four mice (for orogastric infection) $\pm$ SD. PPs, Peyer's patches; MLN, mesenteric lymph node.

Strain	Intravenous infection			Orogastric infection*		
	Days	Spleen	Liver	PPs	Spleen	MLN
WA-314	2	6.03 $\pm$ 0.25	5.96 $\pm$ 0.87	5.91 $\pm$ 0.33	4.01 $\pm$ 0.43	3.85 $\pm$ 0.82
	4	8.46 $\pm$ 0.59	7.02 $\pm$ 0.52			
	5					
WA-314 _{cpdB}	2	5.41 $\pm$ 0.36	3.92 $\pm$ 0.88	5.70 $\pm$ 1.08	3.10 $\pm$ 0.84	5.88 $\pm$ 0.90
	4	7.30 $\pm$ 0.49	6.28 $\pm$ 0.24			
	5					

monas, *Alcaligenes faecalis* and *Y. pestis*) were also found to be deficient in CpdB activity. In agreement with this hypothesis, we discovered that CpdB endows *Y. enterocolitica* with the unusual ability to grow on 2',3'-cyclic nucleotides as the sole source of carbon and energy. Furthermore, the *cpdB* mutant strain was no longer able to grow on this medium, indicating that CpdB is required for growth on 2',3'-cAMP. Young & Miller (1997) recently described a different periplasmic cyclic phosphodiesterase, the 3',5'-cyclic phosphodiesterase of *Y. enterocolitica*. This enzyme was found to be a host-responsive element, being expressed in infected mouse lymphoid tissue but not under *in vitro* culture conditions. The finding that *Yersinia* spp. have high levels of CpdB activity and that other bacterial cyclic phosphodiesterases are implicated in virulence prompted us to clone, characterize and investigate a possible virulence function for the *cpdB* gene of *Y. enterocolitica*.

The *cpdB* gene was cloned by screening a *Y. enterocolitica* O:8 gene bank with the chromogen NPPC. A 3 kb fragment was isolated and subsequently sequenced. This fragment showed an open reading frame with a good Shine-Dalgarno sequence encoding a protein of 68 kDa. The first 24 deduced amino acid residues showed features of a typical prokaryotic signal sequence, as is expected of a secreted protein. The *cpdB* gene of *Y. enterocolitica* showed the highest amino acid homology to *cpdB* genes of *E. coli* and *S. typhimurium* (Liu *et al.*, 1986; Liu & Beacham, 1990). Furthermore, significant homology to 5'-nucleotidases and mosquito apyrase was detected. These enzymes are evolutionarily related, as indicated by several highly conserved amino acid clusters in all three enzymes (Champagne *et al.*, 1995; Zimmermann, 1992). Two of these clusters, which are located in the N-terminal ends of these enzymes, are also present in *Y. enterocolitica* CpdB. It is to be expected that these two regions have a significant role in enzyme function, possibly representing substrate-binding sites. 5'-Nucleotidases have been characterized from different cellular locations and from a wide variety of species ranging from bacteria to vertebrates. These enzymes hydrolyse 5'-ribo- and 5'-deoxyribonucleotides. Periplasmic bacterial 5'-nucleotidase, also known as UDP-sugar hydrolase, degrades UDP-glucose and other nucleotide diphosphate sugars, producing uridine monophosphate and sugar 1-phosphate. The physiological function of bacterial 5'-nucleotidases is probably to provide a carbon source for the cell (Zimmermann, 1992). Mosquito apyrase (ATP-diphosphohydrolase) catalyses the hydrolysis of ATP into AMP, preventing ADP-dependent platelet aggregation in the host and thereby facilitating haematophagy (Champagne *et al.*, 1995).

The finding that *Y. enterocolitica*, which has high CpdB activity, harbours an ERIC sequence in its promoter region led us to investigate a possible function of ERIC in regulating *cpdB* gene expression. ERIC sequences are 126 bp repeat elements that have been found exclusively in transcribed regions of genomes, upstream or downstream of open reading frames. ERIC sequences are

highly conserved, but locations differ among species. This is also the case for the ERIC element of the *cpdB* gene, which is present only in *Y. enterocolitica* strains, and absent in the closely related strains *Y. pestis* and *Y. pseudotuberculosis*. Most ERIC sequences have been found in *E. coli* and *S. typhimurium*, but their discovery in *Yersinia*, *Klebsiella*, *Vibrio*, *Erwinia* and *Xenorhabdus* suggests that they are more widely distributed (Hulton *et al.*, 1991). The ERIC sequence in the *Y. enterocolitica* *cpdB* promoter region, like all ERIC sequences, contains core inverted repeats which can potentially form a stem-loop structure when transcribed. In places where the *Y. enterocolitica* ERIC sequence differs from the consensus sequence, complementary base changes are seen in the stem-loop structure. This, together with the fact that ERIC sequences can be found in both orientations relative to the direction of transcription, indicates that the function is dependent on the secondary structure and not the primary sequence. To date, there have been no reports demonstrating a specific function for ERIC sequences. However, a related repetitive sequence element of *E. coli* and *S. typhimurium*, the REP sequence, has been shown to have specific functions, stabilizing upstream mRNA and influencing gene expression (Newbury *et al.*, 1987a, b), terminating transcription (Gilson *et al.*, 1986) or affecting translational coupling (Stern *et al.*, 1988). We were unable to demonstrate a specific effect on gene expression for the ERIC element in the *cpdB* promoter region.

The cyclic phosphodiesterase of *S. typhimurium* and *E. coli* was shown to be regulated by carbon-source availability. *E. coli* and *S. typhimurium* showed an increase in CpdB activity when grown with glucose, glycerol or succinate as the sole carbohydrate source. Mutants that were unable to synthesize cAMP or CRP showed reduced CpdB activity. CpdB of *Y. enterocolitica* could also be regulated by the cAMP catabolite repression system, since sequences with high similarity to the cAMP-CRP complex were identified between positions 513 and 533. The essential binding element of the cAMP-CRP complex is the TGTGA motif, which is completely conserved. Furthermore, cultures grown with added glucose downregulated *cpdB* expression, indicating that *cpdB* is catabolite-repressed and regulated by the cAMP-CRP complex. The *cpdB* promoters of *S. typhimurium* and *E. coli* belong to a group of promoters that are only weakly modulated by cAMP-CRP. These promoters have a cAMP-CRP-binding site that is very close to the RNA-polymerase-binding site (5–12 bp upstream of the -35 region). This is not the case for *Y. enterocolitica*, in which the TGTGA sequence is located 37 bp upstream of the closest putative -35 hexamer. Downstream of the cAMP-CRP complex, we found sequences with good agreement with the σ^{28} consensus of *E. coli*, which directs transcription of flagellar genes (including flagellin), motility and chemotaxis genes. The σ^{28} factor has been shown to promote RNA polymerase binding to this sequence (Helman, 1991). In *Y. enterocolitica*, the σ^{28} factor was

required for motility but not for fibrillar synthesis or Yop secretion. The promoter regions of the *Y. enterocolitica* *lcrD* and *myfA* genes, which are responsible for the latter two functions, have regions that strongly resemble the σ^{28} consensus sequence. However, these were not recognized by the σ^{28} factor (Iriarte *et al.*, 1995).

Insertional inactivation of the *cpdB* gene of *Y. enterocolitica* showed only a marginal effect on virulence in the orogastric and intravenous mouse infection models. *Y. enterocolitica* strains of biotype 1B are highly pathogenic for mice. These enteric pathogens use Peyer's patches as the port of entry and then disseminate to the spleen and the liver, where they form abscesses. The colonization of Peyer's patches and the initiation of infection, as well as the ability to generate systemic infection, were comparable for wild-type and *cpdB* mutant strains. CpdB does, however, enable *Y. enterocolitica* to metabolize 2',3'-cAMP. This could give yersiniae and other enterobacteria a selective advantage over bacteria deficient in this enzyme activity, in certain ecological niches or in the infection process. The ubiquitous nature of this enzyme further suggests a scavenging role. We therefore propose that CpdB, like other periplasmic phosphatases, is a fitness factor involved in the salvage of nucleotides from the environment.

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