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Genetic basis for the β-haemolytic/cytolytic activity of group B *Streptococcus*

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Summary

Group B streptococci (GBS) express a β-haemolysin/cytolysin that contributes to disease pathogenesis. We report an independent discovery and extension of a genetic locus encoding the GBS β-haemolysin/cytolysin activity. A plasmid library of GBS chromosomal DNA was cloned into *Escherichia coli*, and a transformant was identified as β-haemolytic on blood agar. The purified plasmid contained a 4046 bp insert of GBS DNA encoding two complete open reading frames (ORFs). A partial upstream ORF (*cylB*) and the first complete ORF (*cylE*) represent the 3′ end of a newly reported genetic locus (*cyl*) required for GBS haemolysin/cytolysin activity. ORF *cylE* is predicted to encode a 78.3 kDa protein without GenBank homologies. The GBS DNA fragment also includes a previously unreported ORF, *cylF*, with homology to bacterial aminomethyltransferases, and the 5′ end of *cylH*, with homology to 3-ketoacyl-ACP synthases. Southern analysis demonstrated that the *cyl* locus was conserved among GBS of all common serotypes. Targeted plasmid integrational mutagenesis was used to disrupt *cylB*, *cylE*, *cylF* and *cylH* in three wild-type GBS strains representing serotypes Ia, III and V. Targeted integrations in *cylB*, *cylF* and *cylH* retaining wild-type haemolytic activity were identified in all strains. In contrast, targeted integrations in *cylE* were invariably non-haemolytic and non-cytolytic, a finding confirmed by in frame allelic exchange of the *cylE* gene. The haemolytic/cytolytic activity of the *cylE* allelic exchange mutants could be restored by reintroduction of *cylE* on a plasmid vector. Inducible expression of *cylE*, *cylF* and *cylEF* demonstrated that it is CylE that confers haemolytic activity in *E. coli*. We conclude that *cylE* probably represents the structural gene for the GBS haemolysin/cytolysin, a novel bacterial toxin.

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Introduction

Group B streptococci (GBS) are the leading cause of pneumonia, sepsis and meningitis in human newborn infants (Baker and Edwards, 1995). The great majority of GBS clinical isolates demonstrate β-haemolysis when plated on sheep blood agar, a feature used for their identification in the clinical laboratory (Facklam *et al*., 1979). The GBS β-haemolysin has yet to be isolated, largely because its activity is unstable. High-molecular-weight carrier molecules, such as starch, albumin or Tween 80, are required to preserve haemolytic activity in GBS culture supernatants (Ginsburg, 1970; Marchlewicz and Duncan, 1980). Nevertheless, creation of isogenic GBS transposon mutants exhibiting a non-haemolytic (NH) or hyperhaemolytic (HH) phenotype has facilitated studies to demonstrate that haemolysin is a potent cytotoxin affecting a broad range of host cells. Compared with the parent GBS strains, HH mutants are more injurious and NH mutants less injurious to human lung epithelial (Nizet *et al*., 1996), lung endothelial (Gibson *et al*., 1999), and brain endothelial (Nizet *et al*., 1997a) cells. Moreover, GBS β-haemolysin triggers inducible nitric oxide synthase (iNOS) expression and NO production from macrophages (Ring *et al*., 2000) and is associated with increased mortality in rat, mouse and rabbit models of GBS pneumonia or sepsis (Griffiths and Rhee, 1992; Nizet *et al*., 1997b; Ring *et al*., 1998; Puliti *et al*., 2000). Together, these data suggest that GBS β-haemolysin is a virulence factor in the pathogenesis of neonatal infection that may exert its effect through direct tissue injury or activation of the host inflammatory response (Nizet *et al*., 2000a; Nizet and Rubens, 2000).

Very recently, a major advance towards understanding the genetics underlying GBS β-haemolysin activity was achieved by Spellerberg *et al*. (1999). Using a novel pGh9:ISS1 transposition vector, they generated random mutant libraries of GBS, analysed several NH mutants and identified a genetic locus harbouring eight open reading frames (ORFs) potentially involved in β-haemolysin production. This operon, designated *cyl* with reference to the proven cytolytic activity of the β-haemolysin, contained genes encoding an apparent

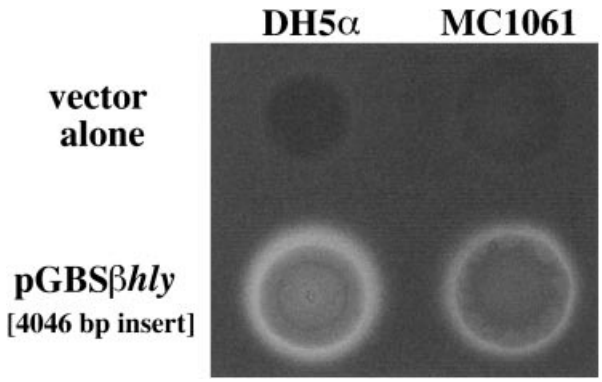

Fig. 1. Identification of a GBS DNA fragment that confers β-haemolysis to *E. coli*.

ABC transport system (*cyl*A and *cyl*B), an acyl carrier protein homologue (*acp*C), additional proteins involved in fatty acid biosynthesis (*cyl*D, *cyl*G and *cyl*Z) and two ORFs of unknown function (*cyl*X and *cyl*E). The available experimental data, however, was insufficient to identify the structural gene for haemolysin/cytolysin itself.

In the present study, we approached the genetic analysis of the GBS haemolysin/cytolysin using a positive selection strategy, identifying GBS genes that confer a β-haemolytic phenotype to *Escherichia coli*. These studies led us to an independent discovery and extension of the *cyl* locus required for GBS haemolysin/cytolysin activity. Targeted mutagenesis, complementation and expression analyses were used to define *cyl* genes necessary for GBS haemolysin/cytolysin activity and sufficient to confer β-haemolysis to *E. coli*. From these data, we conclude that *cyl*E represents the probable structural gene for the GBS haemolysin/cytolysin, a novel bacterial toxin.

Results

Identification of the GBS DNA fragment that confers β-haemolysis to E. coli

A plasmid library of GBS chromosomal DNA fragments was cloned in vector pHPS9 and used to transform *E. coli* strain DH5α. From approximately 5000 colonies screened on SBA, one transformant was found to exhibit notable β-haemolysis after 48 h incubation. The recombinant plasmid from this transformant, pGBSβ*hly*, was purified and used to transform *E. coli* strain MC1061, to which it also conferred a β-haemolytic phenotype (Fig. 1). Restriction analysis demonstrated that pGBSβ*hly* contained an ≈4 kb insert of GBS chromosomal DNA.

DNA sequence analysis and extension of the GBS cyl *locus*

The GBS chromosomal DNA insert from pGBSβ*hly* was analysed and found to represent a 4046 bp product of *Sau*3A1 partial digestion. The entire insert was sequenced and found to contain two complete ORFs, as well as partial ORFs upstream and downstream (Fig. 2). A search for local sequence homology in GenBank using the BLAST algorithm revealed the partial upstream ORF and the first complete ORF to be identical to the 3′ end of the newly reported genetic locus (*cyl*) involved in GBS haemolysin/cytolysin activity (Spellerberg *et al*., 1999). The partial upstream ORF corresponded to the 5′ end of *cyl*B, encoding the transmembrane protein of a typical ABC transport system. The first complete ORF in our GBS DNA fragment corresponded to *cyl*E, encoding a 78.3 kDa protein that does not share homology with other genes in GenBank nor with genes in the group A streptococcal (GAS) genome sequencing project database (http://www.genome.ou.edu/strep.html). The GBS DNA fragment also included a previously unreported ORF, *cyl*F, encoding a 36.6 kDa protein that shares weak homology with bacterial aminomethyltransferases, and the 5′ end of *cyl*H. Subsequently, an ≈4.1 kb *Hin*dIII–*Eco*R1 fragment of GBS COH1 chromosomal DNA, beginning at a *Hin*dIII site within the 5′ end of *cyl*H, was identified and cloned to yield p*cyl*Down, which was used as a template for downstream sequence analysis. These data provided the complete *cyl*H as well as *cyl*I, which encode 42.5 kDa and 36.6 kDa predicted products with homology to 3-ketoacyl-ACP synthases, *cyl*J encoding a 46.6 kDa predicted protein with homology to glycosyltransferases and *cyl*K encoding a 20.5 kDa product

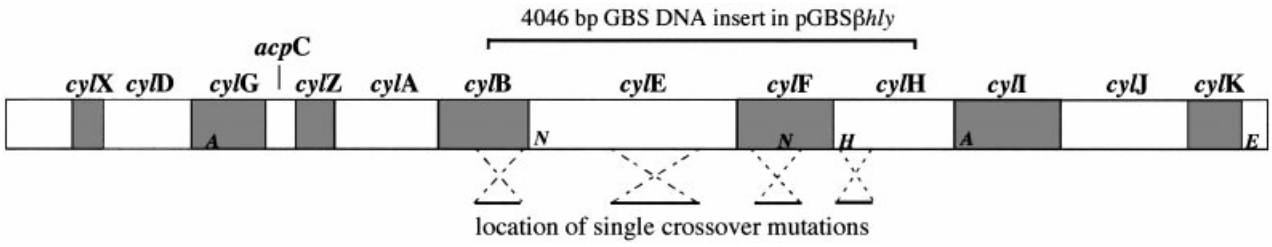

Fig. 2. Map of the complete GBS *cyl* locus involved in haemolysin/cytolysin expression. ORFs *cyl*X–*cyl*E were first reported by Spellerberg *et al.* (1999). Genes targeted for plasmid integrational mutagenesis are shown. Restriction enzyme recognition sites listed: *A*, *Acc*I; *N*, *Nde*I; *H*, *Hin*dIII; *E*, *Eco*RI.

Table 1. Predicted *cyl* gene products.

Gene product	Length (aa)	Mass (kDa)	Homologies (GenBank)	Example of homologous gene	Percentage similarity (% identity)	Region of similarity (aa)
CylE	667	78.3	None	–	–	–
CylF	317	36.7	Aminomethyltransferase	*S. coelicolor gcv*T	41 (24)	24–263
CylH	395	42.5	3-ketoacyl-ACP synthase	*E. coli fab*F	51 (31)	69–378
CylI	331	36.7	3-ketoacyl-ACP synthase	*V. harveyi fab*F	47 (25)	24–173
CylJ	403	46.6	Glycosyltransferase	*B. subtilis yji*C	42 (22)	213–400
CylK	178	20.5	None	–	–	–

without GenBank homologies (Table 1). Interestingly, an acyl carrier protein, *acp*C, and other fatty acid or polyketide synthesis gene homologues (*cyl*D, *cyl*G) are located upstream in the *cyl* locus (Spellerberg *et al.*, 1999). The new sequence data from the present study have been submitted to the DDBJ/EMBL/GenBank databases under accession number AF157015.

RT–PCR analysis shows that cyl*E–K genes represent an extension of the* cyl *operon*

In the earlier study, genes *cyl*X to *cyl*E were found to be co-transcribed (Spellerberg *et al.*, 1999). Our examination of the new *cyl*E–*cyl*K sequence did not reveal strong candidates for transcriptional termination. Therefore, we used reverse transcriptase–polymerase chain reaction (RT–PCR) methods (Gupta, 1999; Nizet *et al.*, 2000b) to test whether these downstream genes were present on the same mRNA as *cyl*X–cylE. Using a reverse primer from the 3′ terminus of *cyl*K, RT–PCR products of predicted size were identified spanning the *cyl*E–*cyl*F, *cyl*F–*cyl*H, *cyl*H–*cyl*I, *cyl*I–*cyl*J and *cyl*J–*cyl*K intergenic regions (data not shown). Coupled with the earlier observations, we conclude that all 13 genes of the *cyl* locus represent an operon.

The cyl *locus is conserved across GBS serotypes*

Southern blot analysis was performed to determine the conservation of *cyl* locus genes among β-haemolytic GBS isolates of different capsular serotypes. Recognition sites for restriction enzyme *Acc*I are found 7491 bp apart within *cyl*I and *cyl*G, whereas recognition sites for *Nde*I are found 2417 bp apart within the 5′ portion of *cyl*E and the 3′ portion of *cyl*F. Chromosomal DNA from eight GBS clinical isolates representing serotypes Ia, Ib, II, III, V and VI were digested with *Acc*I or *Nde*I and probed with a PCR amplicon representing *cyl*EF. Hybridization bands corresponding to

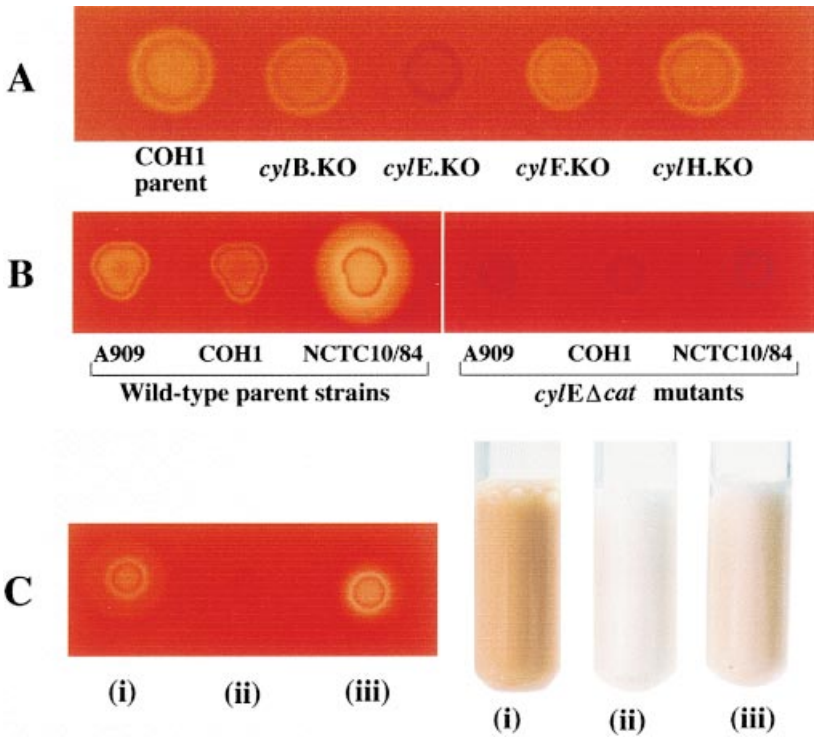

Fig. 3. Effects of targeted mutagenesis of the GBS haemolysin phenotype.
A. Single cross-over targeted mutagenesis of *cyl* locus genes in GBS parent strain COH1.
B. Allelic exchange mutagenesis of *cyl*E gene in three GBS parent strains.
C. Complementation of β-haemolytic and pigment phenotype in *cyl*E allelic exchange mutants (i) NCTC10/84 parent + pDC125 control vector; (ii) NCTC:*cyl*E*Δcat* + pDC125; (iii) NCTC:*cyl*E*Δcat* + pJC10.E.

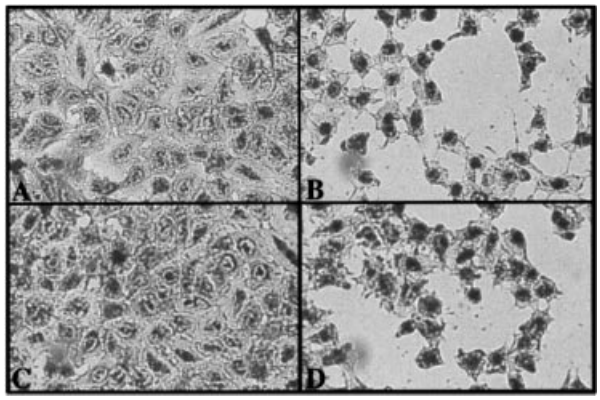

Fig. 4. GBS haemolysin/cytolysin expression and toxicity to A549 human lung epithelial monolayers.
A. Uninfected monolayer.
B. Wild-type GBS strain NCTC10/84.
C. NCTC10/84:*cyl*E.KO.
D. NCTC10/84:*cyl*E.KO [pJC10EF].

the predicted sizes, 7491 bp for *Acc*I and 2417 bp for *Nde*I, were observed for all GBS strains (data not shown). These results indicate that the *cyl* locus is conserved among GBS isolates regardless of capsular serotype.

Targeted plasmid integrational mutagenesis suggests that cyl*E is required for GBS haemolysin/cytolysin activity*

In order to demonstrate the requirement of individual *cyl* locus genes for GBS haemolysin/cytolysin expression, targeted plasmid integrational mutants of *cyl*B, *cyl*E, *cyl*F and *cyl*H were constructed in three GBS parent strains using homologous recombination cassettes cloned into a temperature-sensitive suicide vector. Targeted plasmid integrational mutagenesis of *cyl*E invariably resulted in an NH phenotype in several experiments using all three GBS strains. In contrast, confirmed plasmid integrational disruptions of *cyl*B, *cyl*F and *cyl*H with stable wild-type haemolytic activity were identified for each parent strain. Figure 3A shows a series of confirmed single cross-over plasmid integrational mutants of the *cyl* ORFs compared with GBS parent strain COH1. These data suggested that *cyl*E was required for the GBS haemolysin phenotype, whereas *cyl*B, *cyl*F and *cyl*H were not essential. Of note, many attempts at single cross-over mutagenesis of *cyl*B, *cyl*F and *cyl*H produced a subpopulation of mutants with an NH phenotype (data not shown). Variable results obtained with mutation of the latter ORFs suggested that single cross-over plasmid integration in these genes could occasionally engender mutations in or interfere with transcription of adjacent genes (e.g. *cyl*E). Therefore, to be certain of the essential role of *cyl*E in GBS haemolysin/cytolysin production and to eliminate the danger of polar effects, we studied this ORF further with precise in frame allelic exchange mutagenesis and single gene complementation analysis.

Allelic exchange mutagenesis of cyl*E confirms an essential role in GBS haemolysin/cytolysin activity*

We used a double cross-over allelic exchange mutagenesis strategy to verify the unique requirement of *cyl*E for GBS haemolysin/cytolysin production. Mutants were constructed in which *cyl*E was deleted and replaced precisely in frame by a modified ORF, *cyl*EΔ*cat,* in which the first 596 of 667 codons of *cyl*E are substituted with the chloramphenicol acetyltransferase gene (*cat*). Replacement of *cyl*E with the *cyl*EΔ*cat* yielded GBS mutants that were entirely NH when plated on SBA (Fig. 3B). A seemingly invariant association between GBS haemolytic activity and the bacterium's production of an orange pigment has been documented in the literature (Tapsall, 1987; Nizet *et al*., 1996; Spellerberg *et al*., 1999). We found that NH allelic exchange mutants of *cyl*E exhibited concurrent loss of pigment production.

Reintroduced in trans*,* cyl*E can restore the haemolytic phenotype to* cyl*E allelic exchange knock-outs*

To verify that the allelic exchange mutants of *cyl*E became NH by virtue of specific gene disruption and not through polar effects on downstream transcription, complementation analysis was performed. The NH mutants A909:*cyl*EΔ*cat*, COH1:*cyl*EΔ*cat* and NCTC:*cyl*EΔ*cat* were rendered competent and transformed with complementation vector pJC10.E containing the intact *cyl*E gene derived from the original GBS DNA fragment of pGBSβ*hly*. In all three mutant strains, transformation with pJC10.E resulted in partial restoration of both β-haemolytic activity on SBA and orange pigment production. Complementation *in trans* was confirmed by plasmid purification, restriction analysis and vector-specific PCR amplification. Complementation of the NCTC:*cylE*Δ*cat* mutant phenotypes is shown in Fig. 3C.

The role of cyl *genes in lung epithelial cell injury is confirmed*

We have reported previously that the GBS β-haemolysin is a potent cytotoxin that promotes injury to human lung epithelial cells (Nizet *et al*., 1996). To confirm the correlation of GBS β-haemolysin activity with lung epithelial cell injury, we examined the histological appearance of A549 monolayers exposed to parent strain NCTC10/84, the NH mutant *cyl*E mutant and the NH mutant complemented with *cyl*E on a plasmid vector. After bacterial exposure, the monolayers were stained with the vital dye trypan blue, which stains the nuclei of cells that have lost the membrane pump function to exclude it, and counterstained with eosin to demonstrate cellular morphology. The NH *cyl*E mutant did not show histological

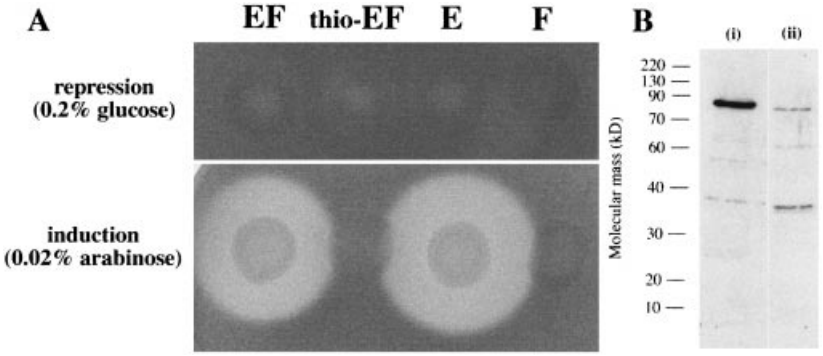

Fig. 5. A. Induced expression of GBS *cyl* genes in *E. coli*. Plasmids used: EF, pCP10.EF; thio-EF, pCP10.thioEF; E, pCP10.E; F, pCP10.F.
B. Confirmation of CylE fusion protein expression in *E. coli* transformed with pCP10.E and induced with 0.02% arabinose. Immunoblot using an antibody versus the C-terminal V5 epitope was performed on sonicate (i) before and (ii) after purification on a nickel-chelating affinity column.

evidence of A549 cell injury, whereas the wild-type strain and the complemented mutant produced severe monolayer damage with cytoplasmic retraction or dissolution and trypan blue-positive staining of nuclei (Fig. 4). These results were corroborated by measuring lactate dehydrogenase (LDH) release from A549 pneumocytes exposed to GBS strains NCTC10/84 and A909 and their respective NH *cyl*E knock-out mutants. In each case, the loss of β-haemolytic activity produced by *cyl*E disruption was associated with the absence of cytolytic activity in A549 cells, and complementation of *cyl*E partially restored cytolytic activity (data not shown). These results confirm the involvement of the *cyl*E gene in the cytolytic as well as the haemolytic activity of GBS.

Expression analysis suggests that cylE *encodes the haemolysin/cytolysin or its precursor*

Targeted mutation and complementation studies in GBS suggested that *cyl*E was likely to encode the GBS haemolysin/cytolysin activity. However, in the pGBSβ*hly* plasmid identified in the β-haemolytic *E. coli* transformant, both *cyl*E and *cyl*F were present as complete ORFs. In order to determine accurately how either or both ORFs are involved in conferring the GBS β-haemolytic phenotype, *cyl*E, *cyl*F and *cyl*EF were amplified by PCR and cloned as fusion proteins in pBAD *E. coli* expression vectors (Invitrogen) under the control of the tightly regulated *ara*BAD promoter. This system was selected to give precisely controlled expression of potentially toxic genes through positive and negative regulation of the *ara*BAD promoter by the product of the *ara*C gene also present on the vectors (Carra and Schleif, 1993; Guzman *et al*., 1995). The resultant constructs were named pCP10.E, pCP10.F and pCP10.EF.

We found that, under *ara*BAD-inducing conditions (0.02% arabinose in SBA media), *E. coli* Top 10 cells harbouring pCP10.EF or pCP10.E, but not pCP10.F, exhibited a pronounced β-haemolytic phenotype after 48 h of incubation (Fig. 5A). Expression products of the predicted size for CylE (pCP10.E), CylF (pCP10.F) and CylEF (pCP10.EF) were confirmed by immunoblot analysis using a primary antibody against the C-terminal V5 epitope of the fusion proteins (data not shown). Under *ara*BAD-repressing conditions (0.2% glucose in SBA media), haemolytic activity in *E. coli* was not observed with any of the constructs. The *cyl*EF PCR product was also cloned in the pBAD system with an N-terminal thioredoxin moiety (pCP10.thio-EF) in an attempt to increase fusion protein solubility. Although inducible expression of the thio–*cyl*EF fusion was confirmed by immunoblot analysis with an antithioredoxin primary antibody, this construct did not confer β-haemolysis to *E. coli* (Fig. 5A). Inducible expression of pCP10.E with robust β-haemolysis was reproduced equally in *E. coli* parent strain MC4100 and its derivative CFP 201 containing a Tn*5* disruption in the *E. coli* cryptic haemolysin gene *she*A (data not shown). Thus, cylE expression does not produce β-haemolysis in *E. coli* by virtue of *she*A induction. Taken together, the data from our inducible expression analysis suggest that *cyl*E encodes the GBS haemolysin/cytolysin or its precursor, and that the native N-terminus of the *cyl*E gene product is required for its β-haemolytic activity.

Nickel-affinity chromatography was used in an attempt to purify further the $(His)_6$-tagged CylE fusion protein under native and denaturing conditions. Although immunoblot analysis of *E. coli* [pCP10.E] lysates demonstrated the expected full-length ≈84 kDa product, the protein fraction eluted from the affinity column revealed instability of the CylE fusion protein with possible ≈70 kDa and ≈40 kDa C-terminal breakdown products (Fig. 5B). Products of similar size were not observed on immunoblot analysis of the CylF expression constructs. Haemolytic activity was not observed in any of the CylE fractions eluted from the column and could not be reconstituted by *post hoc* addition of potential stabilizers such as 1% starch and 3% Tween 80.

Discussion

The GBS haemolysin/cytolysin is an elusive molecule that

appears to play an important role in the pathogenesis of disease in human newborns. Investigations into the nature of the GBS haemolysin/cytolysin have entered a new phase with the recent identification of the *cyl* genetic locus by Spellerberg *et al*. (1999) using a novel random mutagenesis strategy. Our independent discovery and extension of the GBS *cyl* locus via a fundamentally different positive selection approach confirms the essential nature of this now 13 gene operon in GBS haemolysin/cytolysin activity. Further data that we present encompassing allelic exchange mutagenesis, complementation and expression analyses indicate that the *cyl*E gene is both necessary for the GBS haemolysin/cytolysin phenotype and sufficient to confer β-haemolysis to *E. coli*. The simplest and most likely interpretation of these data is that *cyl*E represents the structural gene for the GBS haemolysin/cytolysin or its precursor. If this proves to be true, the GBS haemolysin/cytolysin represents a completely novel type of bacterial toxin.

Expression of the full-length $(His)_6$-tagged recombinant GBS CylE in inducibly β-haemolytic *E. coli* was confirmed by Western blot analysis. A similar construct in which an N-terminal thioredoxin moiety had been added to CylE did not confer haemolysis, suggesting that the native N-terminus of CylE is required for its haemolytic activity. However, in addition to its lack of homology with known bacterial genes, we have not identified any elements of the CylE primary sequence or secondary structure that are suggestive of a well-established mechanism of haemolysin action. CylE is not predicted to have an N-terminal signal sequence, and neither does CylE possess a C-terminal LPXTG sorting signal characteristic for Gram-positive cell wall-anchored proteins (Schneewind *et al*., 1993). Thus, like many bacterial toxins, it is likely that CylE would require specific processing and/or transport mechanisms perhaps encoded by other gene products in the GBS *cyl* locus.

Three of the genes in the upstream region of the GBS *cyl* locus (*cyl*D, *cyl*G and *cylZ*) have significant homologies to enzymes involved in bacterial fatty acid biosynthesis (Spellerberg *et al*., 1999). Our discovery of downstream genes *cyl*H and *cyl*I, each with homology to 3-ketoacyl-ACP synthases, adds to the list of biosynthetic genes that are clustered around an acyl carrier protein gene (*acp*C) in a manner reminiscent of the *fab* gene clusters of *E. coli*, *Vibrio harveyi* and *Bacillus subtilis* (Rawlings and Cronan, 1992; Morbidoni *et al*., 1996; Shen and Byers, 1996). Indeed, the putative haemolysin gene *cyl*E, along with *cyl*F encoding a gene product with weak homology to aminomethyltransferases, appear to be anomalously located amid a fatty acid biosynthesis operon. Interestingly, the 46 kDa haemolysin gene of *Treponema denticola*, an oral spirochaete implicated in periodontitis, possesses homology to genes in a separate family of pyridoxal-phosphate-dependent aminotransferases (Chu *et al*., 1995).

Fatty acylation of specific residues is an essential post-translational modification of some RTX family toxins, including the *E. coli* haemolysin HlyA. It is theorized that the acyl chains on such toxins may provide anchorage points onto the surface of the target cell lipid bilayer or could enhance protein–protein interactions between monomers to bring about oligomerization during pore formation (Stanley *et al*., 1998). A non-haemolytic GBS variant identified after disruption of the *acp*C gene by random ISSI mutagenesis showed a partial restoration of haemolytic activity following reintroduction of the gene on a plasmid vector (Spellerberg *et al*., 1999). This finding suggests that acylation may be required for GBS haemolysin/cytolysin function, and that the non-haemolytic phenotype of the *acp*C mutant did not simply reflect a polar effect on downstream transcription of *cyl*E, the putative GBS haemolysin precursor gene. It is important to note that CylE does not share homology to the RTX family of haemolysin/cytotoxins, and that expression of β-haemolysis in *E. coli* was accomplished by cloning of *cyl*E in the absence of *acp*C. These data contradict the hypothesized requirement for acylation or indicate that sufficient modifications can be accomplished by other gene products in the *E. coli* background.

Our targeted mutagenesis data suggest that the genes flanking *cyl*E in the locus, *cyl*B, cylF and *cyl*H, are not themselves required for the haemolysin/cytolysin phenotype. CylB appears to represent the transmembrane component of an ABC-type transporter, not uncommonly associated with the export of bacterial cytotoxins. If the CylA–B transporter is indeed normally involved in GBS haemolysin/cytolysin export as suggested previously (Spellerberg *et al*., 1999), it is possible that the haemolysin/cytolysin may be able to scavenge other transporters to reach the cell surface.

The invariant association of loss of orange pigment production with loss of β-haemolytic activity among our GBS *cyl*E mutants corroborates earlier observations of the tight linkage of these two phenotypes (Tapsall, 1987; Nizet *et al*., 1996; Spellerberg *et al*., 1999). The orange pigment of GBS possesses characteristics of a β-carotenoid (Merritt *et al*., 1976) but loses spectral absorption properties in the absence of the high-molecular-weight carriers (e.g. albumin and starch) required for stabilization of haemolytic activity (Tapsall, 1986). It is possible that the GBS haemolysin itself is a pigmented molecule or that specific genes in the *cyl* locus are essential for the biosynthesis of both haemolysin and pigment. Alternatively, expression of a pigmented phenotype could be a post-translational effect of the GBS haemolysin/cytolysin protein on the bacterial cell. Importantly, we did not find *E. coli* transformed with GBS *cyl*E,

*cyl*F or *cyl*EF to exhibit a pigmented phenotype. The legiolysin (*lly*) gene of *Legionella pneumophila* confers both a haemolytic phenotype and brown pigmentation when cloned in *E. coli* (Wintermeyer *et al*., 1991); however, when *lly* is disrupted in *L. pneumophila*, only pigmentation is lost, whereas haemolysis is unaffected (Wintermeyer *et al*., 1994). A genetically uncharacterized single insertion Tn*4351* mutant of *Porphyromonas gingivalis* was also found recently to be deficient in both pigmentation and haemolytic activities (Takada and Hirasawa, 1998).

An alternative explanation for our findings that we have considered is that *cyl*E, rather than representing the gene for the GBS haemolysin/cytolysin precursor, encodes a protein that is (i) absolutely required for expression, processing or export of a still-to-be-discovered GBS haemolysin protein; and (ii) coincidentally capable of activating a cryptic or silent *E. coli* β-haemolysin. Indeed, a number of recent reports have identified cloned gene products from heterologous bacterial species that can induce expression of the 34 kDa cryptic *E. coli* haemolysin ClyA (also known as SheA). To date, all such inducer genes are from Gram-negative bacterial species and have strong sequence similarities to genes for known transcriptional activators. Examples are *fnr*P of *Pasteurella haemolytica* (Uhlich *et al*., 1999) and *hly*X of *Actinobacillus pleuropneumoniae* (MacInnes *et al*., 1990), both of which share strong homology with the gene for the native *E. coli* inducer FNR, and *sly*A of *Salmonella typhimurium* (Ludwig *et al*., 1995), which has strong homology to the gene for the native *E. coli* inducer MprA (del Castillo *et al*., 1997). We found that inducible expression of the GBS CylE fusion protein conferred robust β-haemolysis to the *she*A-negative mutant CFP 201, ruling out induction of the only known *E. coli* cryptic haemolysin as an explanation for our results. Moreover, GBS CylE shares no homology with any known transcriptional activator, nor is it predicted by sequence analysis to possess a helix–turn–helix DNA-binding motif common to FNR-like regulators (Oscarsson *et al*., 1999).

The β-haemolytic phenotype in all wild-type GBS or pJC10.E-complemented GBS *cyl*E knock-outs is readily observed on SBA plates within 16 h of incubation at 37°C. In *E. coli* transformed with plasmids bearing GBS *cyl*E, the phenotype requires 48 h to develop. One explanation for this phenomenon is that the processing or transport mechanisms for the secretion of CylE are absent in the *E. coli* host, and the phenotype is seen as dying cells release their cytoplasmic contents into the surrounding media. This conclusion is consistent with our finding that haemolytic activity could not be detected in supernatants from induced log-phase cultures of *E. coli* containing any of the *cyl*E constructs, even in the presence of known stabilizers of the GBS haemolysin/cytolysin such as starch or Tween 80 (data not shown). Similar observations were made for heterologous expression of the *Haemophilus ducreyi* haemolysin genes *hhd*A *and hhd*B and the *Prevotella melaninogenica* haemolysin gene *phy*A in *E. coli* (Allison and Hillman, 1997; Dutro *et al*., 1999). This pattern is in contrast to the release of induced *E. coli* cryptic haemolysin ClyA, which is confined to the logarithmic growth phase, suggesting that the protein is secreted by a specific transport mechanism and not unspecifically freed upon lysis of stationary phase bacteria (Ludwig *et al*., 1999).

Although expression of recombinant CylE has allowed us to purify quantities of the full-length fusion protein, problems with instability of haemolytic activity that have complicated several earlier studies of the GBS haemolysin/cytolysin persist. When separated from the complex macromolecular environment of a bacterial colony on a blood agar plate, or without the presence of substitute stabilizer molecules such as starch, albumin or Tween 80, GBS haemolytic activity is irreversibly lost (Ginsburg, 1970; Marchlewicz and Duncan, 1980; Dal and Monteil, 1983). The functional instability of the full-length CylE suggests that the high-molecular-weight stabilizers allow the protein to adopt a conformation that permits interaction with the target cell membrane. In GBS, the stabilizer function may be carried out by one or more cell surface components, as contact between the bacteria and/or bacterial fragments with target red blood cells is required for lysis (Platt, 1995). In addition to functional instability, we found that CylE expressed in *E. coli* broke down into smaller protein species and that the concentration of a major ≈ 40 kDa C-terminal breakdown product increased during chromatographic purification, whether performed under native or denaturing conditions. We speculate that conformational and structural instability of the haemolysin/cytolysin may be a mechanism by which the GBS protects itself against potential damaging effects that could result from high local concentrations of the toxin.

Finally, it cannot be definitively excluded that *cyl*E is not the final haemolysin product but is a biosynthetic enzyme that modifies a cellular metabolite, common to GBS and *E. coli*, to generate the active haemolytic moiety. To our knowledge, all bacterial β-haemolysins identified to date are pore-forming or membrane-destabilizing protein toxins. Further arguing against the counterhypothesis is the observation that GBS haemolytic activity is sensitive to proteases (Marchlewicz and Duncan, 1981), making it likely that a specific GBS protein product exerts the membrane-damaging action.

Our independent discovery and extension of the GBS *cyl* locus has confirmed the essential role of this operon in GBS haemolysin/cytolysin activity and has highlighted the novel gene *cyl*E as both necessary for the phenotype in GBS and sufficient to confer the phenotype to *E. coli*. We are optimistic that continued investigations of the GBS *cyl*

Table 2. Bacterial strains and plasmids.

Bacterial strain/plasmid	Description	Reference
Group B streptococci		
COH1	Heavily encapsulated serotype III from blood of septic neonate	Wessels *et al.* (1991)
A909	Serotype IA from blood of septic neonate	Madoff *et al.* (1991)
NCTC10/84	Serotype V from blood of septic neonate (also known as 1169-NT1)	Wilkinson (1977)
E. coli		
DH5α	*end* A1 *hsd* R17 ($r_k^-m_k^+$) *sup* E44 *thi*-1 *rec* A1 *gyr* A (Nal^R) *Rel* A1 Δ(*lac* ZYA-*arg* F) U169 (ϕ80*dLac* Δ(*lac* Z)M15)	Woodcock *et al.* (1989)
MC1061	F^- *ara* D139 Δ(*ara* ABC-*leu*)7696 Δ(*lac*) X74 *gal* U *gal* K *Hsd* R2 ($r_k^-m_k^+$) *mcr* B1 *rps* L (Str^R)	Wertman *et al.* (1986)
Top 10	F^- *mcr* A Δ(*mrr-hsd* RMS-*mcr* BC) ϕ80*lac* ZΔM15 Δ(*lac*) X74 *deo* R *rec* A1 *ara* D139 Δ(*ara-leu*)7697 *gal* U *gal* K *rps* L (Str^R) *end* A1 *nup* G	Grant *et al.* (1990)
MC4100	F-*ara*D139 Δ(*argF-lac*)U169 *rps*L150 *rel*A1 *thi*A *flb*B5301 *deo*C1 *pts*F25 *rbs*R	del Castillo *et al.* (1997)
CFP 201	MC4100 *she*A::Tn*5*-2.1 (lacking cryptic haemolysin activity)	del Castillo *et al.* (1997)
Plasmid		
Chromosomal library		
pHPS9	*E. coli*/*Bacillus* shuttle vector, Cm^R, Em^R, lacZα	Haima *et al.* (1990)
pGBSβ*hly*	pHPS9, containing 4046 bp *Sau*3A1 fragment of GBS strain COH1 chromosomal DNA, confers β-haemolytic phenotype to *E. coli*	This study
Chromosomal walking		
pBluescript II	ColE1 ori, Amp^R, *lac*Zα	Stratagene
p*cyl*Down	pBluescript II with 4.1 kb fragment of GBS chromosomal DNA from *Hin*dIII site in *cyl*H downstream to *Eco*RI site	This study
Targeted plasmid integrational mutagenesis		
pCR2.1-topo	ColE1 ori, Amp^R, Kn^R, *lac*Zα	Invitrogen
pVE6007Δ	Temperature-sensitive derivative of pWV01, Cm^R	Maguin *et al.* (1992)
p*cyl*B.KO	pVE6007Δ + intragenic PCR fragment of *cyl*B (codons 158–265)	This study
p*cyl*E.KO	pVE6007Δ + intragenic PCR fragment of *cyl*E (codons 245–518)	This study
p*cyl*F.KO	pVE6007Δ + intragenic PCR fragment of *cyl*F (codons 27–178)	This study
p*cyl*H.KO	pVE6007Δ + intragenic PCR fragment of *cyl*H (codons 95–183)	This study
Allelic exchange mutagenesis of *cyl*E gene		
pGEM-5Zf(+)	f1 ori, Amp^R, *lac*Z	Promega
pHY304	$Temp^S$ pVE6007Δ derivative, Em^R + *lac*Zα/MCS of pBluescript	Gift from Dr H. H. Yim
pACYC184	rep(p15A), Cm^R, Tet^R	Rose (1988)
pGEM*cyl*	pGEM-5Zf(+) with ≈4 kb GBS DNA fragment from pGBSβ*hly*	This study
pGEM*cyl*EΔ*cat*	*In vivo* PCR recombination derivative of pGEM*cyl* with *cat* replacing first 596 codons of the GBS *cyl*E gene, Amp^R, Cm^R	This study
p*cyl*EΔ*cat*	pHY304 containing *cyl*EΔ*cat* gene and flanking DNA, Em^R, Cm^R	This study
Complementation analysis		
pDC123	*E. coli*/streptococcal shuttle vector, JS-3 replicon, Cm^R	Chaffin and Rubens (1998)
pDC125	*E. coli*/streptococcal shuttle vector, JS-3 replicon, Em^R	Chaffin *et al.* (1998)
pJC10.EF	pDC125 with GBS DNA fragment from pGBSβ*hly* (*cyl*E + *cyl*F)	This study
pJC10.E	pJC10.EF derivative with *cyl*F deleted by inverse PCR	This study
Expression analysis		
pBAD-TOPO	ColE1 ori, *ara*C, *ara*BAD promoter, Amp^R,V5 epitope, 6xHis	Invitrogen
pBADthio-TOPO	pBAD-TOPO plus N-terminal HP-thioredoxin	Invitrogen
pCP10.EF	*cyl*E–F cloned as in frame fusion in pBAD-TOPO	This study
pCP10.thio-EF	*cyl*E–F cloned as in frame fusion in pBADthio-TOPO	This study
pCP10.E	*cyl*E cloned as in frame fusion in pBAD-TOPO	This study
pCP10.F	*cyl*F cloned as in frame fusion in pBAD-TOPO	This study

locus and the molecular basis of GBS haemolysin/cytolysin expression will shed important new light on the role of this unique cytotoxin in the pathogenesis of neonatal infection.

Experimental procedures

Bacterial strains, culture conditions and transformation

Bacterial strains are listed in Table 2. GBS were grown in Todd–Hewitt broth (THB) or on trypticase soy agar + 5% sheep's blood (SBA). For antibiotic selection, 5 μg ml^{-1} chloramphenicol (Cm) or erythromycin (Em) was added to the media. *E. coli* were grown in Luria broth (LB) or on LB agar; antibiotic selection used 50 μg ml^{-1} ampicillin (Amp), 5 μg ml^{-1} Cm or 300 μg ml^{-1} Em. GBS were made competent for transformation by growth in THB + 0.6% glycine (Framson *et al.*, 1997), and *E. coli* were rendered competent by standard methods (Dower *et al.*, 1988). Plasmids were introduced by electroporation (Eppendorf 2510, 1500 V), with recovery in THB + 0.25 M sucrose for 1–2 h before antibiotic selection on agar media.

Chromosomal library of GBS DNA

High-molecular-weight chromosomal DNA of GBS strain COH1 was isolated as described previously (Kuypers *et al.*,

1989), partially digested with *Sau*3AI, and fragments from 3.0 to 8.0 kb in size were purified by 0.6% TAE–agarose gel electrophoresis. These fragments were ligated into *Bam*HI-cut pHPS9 and used to transform *E. coli* DH5α to Cm^R for screening on SBA. The recombinant plasmid pGBSβ*hly* was purified from a β-haemolytic *E. coli* transformant.

DNA sequence analysis

Plasmid pGBSβ*hly* contained a 4046 bp fragment of GBS strain COH1 chromosomal DNA that was completely sequenced using the dideoxy dye termination method (Perkin-Elmer Cetus) at the Molecular Biology Core Facility of the UCSD Center for AIDS Research. An ≈4.1 kb *Hin*dIII–*Eco*R1 fragment of GBS COH1 chromosomal DNA, beginning at a *Hin*dIII site within *cyl*H (position 3624 of the pGBSβ*hly* insert), was recognized by Southern analysis and recovered from a *Hin*dIII–*Eco*RI library of COH1 DNA cloned in pBluescript II by PCR screening. This plasmid, p*cyl*Down, was used as a template for sequence analysis of downstream genes in the *cyl* operon. The BLAST program service of the NCBI/NIH (Altschul *et al*., 1990) was used as a local alignment tool to compare the GBS sequence with those in the GenBank databases.

Transcriptional analysis

For RT–PCR analysis, GBS strain COH1 was grown to an OD_{600} of 0.6–0.7. Bacterial pellets were lysed in Tris–EDTA buffer containing lysozyme (5 mg ml^{-1}) and mutanolysin (1 U μl^{-1}), and total RNA was isolated using the RNeasy midi protocol (Qiagen). RNA samples were digested at 37°C for 1 h using RNase-free DNase I (Roche) to remove contaminating DNA. Reverse transcription was performed using Superscript II (Gibco) and a reverse primer that anneals at the 3′ end of *cyl*K. PCR products were subsequently amplified with specific paired *cyl* primers spanning the intergenic regions (e.g. *cyl*E 5′ fwd and *cyl*F 3′ rev). Control reactions omitting the reverse transcription step were used to confirm the absence of contaminating chromosomal DNA.

Southern analysis

Chromosomal DNA was isolated from GBS strains representing a spectrum of capsular serotypes. Ten micrograms of each preparation was digested to completion with either *Acc*I or *Nde*I, separated by 0.8% TAE–agarose gel electrophoresis and transferred to a nylon filter by the Southern method. A digoxigenin-labelled probe of a PCR amplicon comprising *cyl*EF was prepared using a Genius kit (Boehringer Mannheim). The probe was hybridized to the target filters under standard conditions, and the probe-positive bands were visualized by chemiluminescence.

Targeted plasmid integrational mutagenesis

PCR was used to amplify intragenic fragments from *cyl*B, *cyl*E, *cyl*F and *cyl*H. PCR products were recovered by T–A cloning in the vector pCR2.1-TOPO (Invitrogen) and then cloned by *Hin*dIII–*Xba*I digestion into the temperature-sensitive vector pVE6007Δ. The resultant 'knock-out plasmids' were introduced into wild-type GBS by electroporation, and Cm^R transformants were identified at the permissive temperature for plasmid replication (30°C). Single cross-over Campbell-type chromosomal insertions were selected by shifting to the non-permissive temperature (37°C) while maintaining Cm selection (Yim and Rubens, 1998; Nizet *et al*., 2000a). Haemolytic phenotype was determined on SBA + Cm at 37°C. Fidelity of site-directed recombination events was confirmed by PCR.

Allelic exchange mutagenesis

The ≈4.0 kb fragment of GBS *cyl* DNA from pGBSβ*hly* was subcloned into pGEM-5Zf(+) (Promega) to give vector pGEM*cyl*. Inverse PCR was performed on pGEM*cyl* using (i) a reverse primer from immediately upstream of the *cyl*E start codon; and (ii) a forward primer from near the 3′ end of *cyl*E; these primers were designed with 25 bp 5′ extensions corresponding to the start and end of the chloramphenicol acetylase (*cat*) gene respectively. The resultant PCR product, with the majority of *cyl*E deleted, was used to transform *E. coli* DH5α together with an ≈650 bp PCR amplicon of the complete *cat* gene from pACYC184. *In vivo* recombination events were identified by screening for DH5α exhibiting Amp^R + Cm^R, and the plasmid construct pGEM-*cyl*EΔ*cat* was verified by direct sequencing to possess a precise in frame substitution of the initial 596 (of 667) codons of *cyl*E with the *cat* gene. The mutated *cyl*EΔ*cat* gene was subcloned as an *Nde*I–*Hin*dIII fragment with 122 bp of upstream *cyl*B sequence and 235 bp of downstream *cyl*F sequence to the temperature-sensitive Em^R vector pHY304 to produce knock-out vector p*cyl*EΔ*cat.* GBS was transformed with p*cyl*EΔ*cat*, and single recombination events were identified at 37°C under Em selection. Selection was relaxed by serial passage at 30°C without antibiotics, and double cross-over events were identified as GBS exhibiting Cm^R but Em^S. Allelic exchanges of *cyl*E with *cyl*EΔ*cat* were confirmed by (i) PCR using *cat* primers with upstream and downstream *cyl* locus primers; and (ii) the absence of wild-type *cyl*E amplification.

Complementation analysis

The 4.0 kb+ fragment of GBS DNA containing *cyl*E + *cyl*F was cut from pGBSβ*hly* with *Sal*I–*Sma*I and cloned into *Sal*I–*Eco*RV-cut pDC123. The GBS DNA fragment was cut from this intermediate construct with *Sal*I–*Sph*I and cloned into *Sal*I–*Sph*I-cut pDC125 to create the Em^R vector pJC10.EF (8278 bp). Using pJC10.EF as a template, a reverse primer was chosen from the 5′ end of *cyl*F and a forward primer was chosen from the 5′ end of *cyl*H and used to amplify (Expand system, Boehringer Mannheim) an inverse PCR product that deleted the majority of *cyl*F. The PCR product was gel purified, the ends polished with T4 DNA polymerase and self-ligated. The ligation reaction was digested with *Dpn*I to eliminate any remaining methylated pJC10.EF template and used to transform MC1061 to Em^R.

The resultant complementation vector pJC10.E (6906 bp) was purified from an *E. coli* transformant and confirmed by restriction analysis. Plasmid pJC10.E was used to transform GBS *cyl*E knock-out mutants, and transformants were screened on SBA for haemolytic phenotype.

Lung epithelial cell cytotoxicity assays

Cytotoxicity of GBS to A549 lung epithelial cell (ATCC) monolayers was documented using a chamber slide trypan blue dye exclusion assay. GBS (5×10^7 cfu) were added to monolayers of $\approx 1 \times 10^5$ A549 cells (multiplicity of infection = 500), incubated for 90 min at 37°C, and the monolayers were stained and photographed as described previously (Gibson *et al*., 1999). Injury to A549 cells was corroborated by a microtitre plate assay of LDH release (Nizet *et al*., 1996).

Expression analysis

PCR products of *cyl*E, *cyl*F and *cyl*EF were T–A cloned into pBAD-TOPO (Invitrogen) to create pCP10.E, pCP10.F and pCP10.EF. The constructs eliminated the start and stop codons to create an in frame fusion of the ORFs to a 5′ leader peptide and a 3′ V5 epitope and $(His)_6$-tag. The *cyl*EF product was also T–A cloned into pBAD/Thio-TOPO. These vectors were used to transform *E. coli* Top 10, MC4100 or CFP 201 cells to Amp^R, and haemolytic phenotype was assessed on SBA plates containing either 0.2% glucose or 0.02% arabinose for repression or induction of the *ara*BAD promoter respectively. Recombinant CylE was analysed further in protein fractions obtained from 50 ml arabinose-induced Top 10 [pCP10.E] cultures. Fractions were prepared by lysozyme (100 μg ml^{-1}) treatment and sonication of cell pellets in PBS containing 10 mM imidazole and a protease inhibitor cocktail for bacterial cell extracts (Sigma). The lysate was applied to a 2 ml HiTrap nickel-chelating column (Pharmacia), and the $(His)_6$-tagged proteins eluted from a gradient at a concentration of 100 mM imidazole. Expression of CylE, CylEF and CylF fusion proteins was confirmed by SDS–PAGE and immunoblot analysis using an antibody directed against the C-terminal V5 epitope.

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