

## Characterization of a Pore-Forming Cytotoxin Expressed by *Salmonella enterica* Serovars Typhi and Paratyphi A

Jan Oscarsson,<sup>1</sup> Marie Westermarck,<sup>1</sup> Sven Löfdahl,<sup>2</sup> Björn Olsen,<sup>3,4</sup> Helena Palmgren,<sup>3</sup> Yoshimitsu Mizunoe,<sup>5</sup> Sun Nyunt Wai,<sup>1,5</sup> and Bernt Eric Uhlin<sup>1\*</sup>

Department of Molecular Biology<sup>1</sup> and Department of Infectious Diseases,<sup>3</sup> Umeå University, S-90187 Umeå, Swedish Institute for Infectious Disease Control, S-17182 Solna,<sup>2</sup> and Department of Infectious Diseases, Länssjukhuset (LSK), S-39185 Kalmar,<sup>4</sup> Sweden, and Department of Bacteriology, Faculty of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan<sup>5</sup>

Received 19 February 2002/Returned for modification 15 April 2002/Accepted 2 July 2002

**Cytolysin A (ClyA) is a pore-forming cytotoxic protein encoded by the *clyA* gene that has been characterized so far only in *Escherichia coli*. Using DNA sequence analysis and PCR, we established that *clyA* is conserved in the human-specific typhoid *Salmonella enterica* serovars Typhi and Paratyphi A and that the entire *clyA* gene locus is absent in many other *S. enterica* serovars, including Typhimurium. The gene products, designated ClyA<sub>STy</sub> and ClyA<sub>SPa</sub>, show ≥90% amino acid identity to *E. coli* cytolysin A, ClyA<sub>EC</sub>, and they are immunogenically related. The *Salmonella* proteins showed a pore-forming activity and are hence functional homologues to ClyA<sub>EC</sub>. The chromosomal *clyA*<sub>STy</sub> gene locus was expressed at detectable levels in the serovar Typhi strains S2369/96 and S1112/97. Furthermore, in the serovar Typhi vaccine strain Ty21a, expression of *clyA*<sub>STy</sub> reached phenotypic levels, as detected on blood agar plates. The hemolytic phenotype was abolished by the introduction of an in-frame deletion in the *clyA*<sub>STy</sub> chromosomal locus of Ty21a. Transcomplementation of the mutant with a cloned *clyA*<sub>STy</sub> gene restored the hemolytic phenotype. To our knowledge, Ty21a is the first reported phenotypically hemolytic *Salmonella* strain in which the genetic determinant has been identified.**

Pathogenic bacteria produce many substances that are directly or indirectly toxic to host cells, and such toxins often play a central role in the pathogenesis of microbial disease (9). *Salmonella enterica* colonizes and infects a wide range of different organisms and causes a broad spectrum of diseases, such as gastroenteritis and bacteremia, as well as life-threatening conditions, such as typhoid fever (32). Although some *S. enterica* serovars, including Typhimurium, can infect a broad range of hosts, other serovars, such as Typhi and Paratyphi A (humans) and Dublin (cattle), have a narrow host range. The molecular bases for the host restriction, however, are not well understood. Virulent serovars possess an array of genes that encode proteins required for invasion and that are secreted by a specialized type III secretion apparatus (10). Cytotoxic activity towards cultured macrophages of *Salmonella*, independent of host restriction, is evident (6), and it has also been established that serovar Typhimurium and serovar Typhi induce macrophage apoptosis via a mechanism involving the invasin SipB (17, 29). Up to now, however, there has been no experimental evidence for expression of an RTX-like toxin or cytolysin in *Salmonella*, although cytotoxic activity associated with a cholera toxin (CT)-like toxin and an *Escherichia coli* heat-labile enterotoxin (LT-I)-like toxin has been reported (7, 31). Members of the RTX toxin family, and especially the HlyA hemolysin of uropathogenic *E. coli*, are among the most thoroughly studied cytolytic toxins of gram-negative bacteria (3, 48, 49). Using a model for experimental infections in mice, it has been established that HlyA is directly involved in the pathogenesis of *E. coli* infection (14). ClyA (also called HlyE

or SheA), a 34-kDa protein, is the prototype of a growing family of non-RTX cytolysins that was first identified in *E. coli* (8, 13, 35; B. E. Uhlin and Y. Mizunoe, abstract from the Keystone Symposia on Molecular and Cellular Biology: Molecular Events in Microbial Pathogenesis, J. Cell. Biochem. **18A**(Suppl.):71, 1994). X-ray crystallography has shown that ClyA has unusual structural features and does not resemble any previously studied cytotoxin (47). In contrast to RTX toxins, ClyA does not require calcium for activity, nor does it contain the conserved fatty acylation sites typical of RTX toxins or a C-terminal secretion signal (8, 34). Purified *E. coli* cytolysin A (ClyA<sub>EC</sub>) protein has been shown to have a cytotoxic and cell-detaching activity towards murine-derived macrophage-like cells (34). This effect caused apoptosis in the macrophage-like cells (22), and in addition, ClyA<sub>EC</sub> was recently shown to act by forming pores in target membranes (25, 34, 47). Expression of the *clyA* gene is silenced in nonpathogenic *E. coli* K-12 laboratory strains by the abundant nucleoid protein H-NS (50). Thus, in H-NS-deficient *E. coli* K-12 derivatives, expression of ClyA is derepressed, resulting in cytotoxicity towards cultured mammalian cells, e.g., HeLa cells (Uhlin and Mizunoe, J. Cell. Biochem. **18A**(Suppl.):71, 1994; J. M. Gómez-Gómez, J. Blazquez, F. Baquero, and J. L. Martínez, Letter, Mol. Microbiol. **19**:909-910, 1996). Furthermore, phenotypic expression of ClyA is observed in strains carrying any of the genes *slyA*, *mprA*, and *hlyX* on plasmids (8, 13, 25, 26, 35). Recent genome sequence analysis of serovar Typhi CT18 and serovar Typhimurium LT2 revealed the presence of an open reading frame, highly homologous to *clyA*<sub>EC</sub>, in serovar Typhi CT18, which was apparently absent in the LT2 strain analyzed (27, 39). We have screened different *S. enterica* serovars for the presence and expression of *clyA*, and here we report that the gene locus encoding ClyA cytotoxin is con-

\* Corresponding author. Mailing address: Department of Molecular Biology, Umeå University, S-90187 Umeå, Sweden. Phone: 46-90-7856731. Fax: 46-90-772630. E-mail: bernt.eric.uhlin@molbiol.umu.se.

TABLE 1. Plasmids used in this work

Plasmid	Relevant characteristic(s)	Reference or source
pGEM-T Easy	T vector for cloning of PCR fragments; Cb <sup>r</sup>	Promega
pJON80	1.1-kb subclone of <i>chyA</i> <sub>S<sub>Pa</sub></sub> from serovar Paratyphi A S3068/99 in pGEM-T Easy	This work
pJON94	1.1-kb subclone of <i>chyA</i> <sub>S<sub>Ty</sub></sub> from serovar Typhi Ty21a in pGEM-T Easy	This work
pJON97	1.4-kb subclone of <i>chyA</i> <sub>S<sub>Ty</sub></sub> from serovar Typhi S2369/96 in pGEM-T Easy	This work
pJON103	<i>chyA</i> <sub>S<sub>Ty</sub></sub> in-frame deletion in pKO3	This work
pKO3	Gene replacement vector; Cm <sup>r</sup>	24
pMWK21	1.1-kb subclone of <i>chyA</i> <sub>S<sub>Ty</sub></sub> from serovar Typhi S2369/96 in pGEM-T Easy	This work
pMMkatF2	4.2-kb subclone of <i>rpoS</i> <sub>EC</sub> in pAT153	30
pPM103	Gene replacement vector; Tc <sup>r</sup>	28
pUC4K	Kanamycin resistance gene cartridge plasmid; Kan <sup>r</sup>	46
pUC18	Cloning vector; Cb <sup>r</sup>	55
pYMZ71	1.3-kb subclone of <i>chyA</i> <sub>EC</sub> in pUC18	This laboratory
pYMZ80	1.6-kb subclone of <i>chyA</i> <sub>EC</sub> in pUC18	This laboratory
pYMZ81	1.6-kb subclone of <i>chyA</i> <sub>EC</sub> in pUC18	This laboratory
pYMZ103	<i>chyA</i> <sub>EC</sub> :: <i>kan</i> in pUC18	This work

served in typhoid serovars while it is absent in many other serovars, including Typhimurium. As judged by our experimental findings, the *Salmonella* proteins showed a pore-forming activity, and hence they are the first examples of such a factor expressed by *Salmonella*.

(A preliminary account of part of this work has been presented [37]).

#### MATERIALS AND METHODS

**Bacterial strains and culture media.** Serovar Typhimurium LT2 strain GT3258 was obtained from Dan Andersson, Department of Bacteriology, Swedish Institute for Infectious Disease Control, Solna, Sweden. Serovar Typhimurium SVA16 was isolated from a domesticated French quail (*Coturnix coturnix*). Serovar Typhimurium 4576 is a nonsubtypeable, highly antibiotic-resistant clinical isolate acquired in Ethiopia by a Swedish tourist. Serovar Havana St 63/1992 was isolated from animal feed ingredients in Sweden. Serovar Havana SR 15 was isolated from an Antarctic fur seal (*Arctocephalus gazella*) (38). Serovar Enteritidis PT4 was isolated from a Gentoo penguin (*Pygoscelis papua*) (33). Serovar Enteritidis 2894 is a human isolate of subtype PT4 acquired in Greece by a Swedish tourist. Serovar Newport Arg6, serovar Anatum Arg10, and serovar St. Paul Arg11 are all Argentinean human isolates and a kind gift from the Department of Microbiology, Hospital Rawson, Córdoba, Argentina. Serovar Dublin 2229 has been described previously (53). Serovar Typhi S2369/96, S1112/97, S2060/97, S1735/97, S1087/98, and S0200/99 and serovar Paratyphi A S3068/99 were isolated from clinical patients in Sweden who had all acquired typhoid fever abroad at different times and places and hence are epidemiologically unrelated. Ty2 is a wild-type strain of serovar Typhi isolated in 1916. Ty21a is a virulence-attenuated derivative of Ty2 that is used in a human live vaccine against typhoid fever (Vivotif, Berna, Italy) (12). JON42 is Ty21a  $\Delta$ *chyA*. The *E. coli* strains YMZ16 and YMZ19 are derivatives of MC1061 (5) and BEU616 (MC1061 *hns*::*cat*) (50), respectively, that have a kanamycin cassette inserted into the *chyA* locus. DH5 $\alpha$  (*recA1*) (15) was used in cloning experiments. The bacterial strains were grown aerobically at 37°C in Luria-Bertani (LB) broth or on LB broth solidified with 1.5% (wt/vol) agar. Unless stated otherwise, blood agar plates consisted of 5% horse erythrocytes solidified with 1% (wt/vol) Columbia agar (base) (Merck), which, according to the manufacturer, contains 2.3% (wt/vol) special nutrient substrate. When needed, antibiotic selection was carried out using 50  $\mu$ g of carbenicillin ml<sup>-1</sup>, 50  $\mu$ g of kanamycin ml<sup>-1</sup>, 15  $\mu$ g of tetracycline ml<sup>-1</sup>, or 10  $\mu$ g of chloramphenicol ml<sup>-1</sup>.

**Genetic techniques and sequence analysis.** Standard procedures were used in all general molecular applications (42). Generalized bacteriophage P1 transduction was performed as described previously (52). Oligonucleotides were made on an Applied Biosystems 394 synthesizer or obtained from DNA Technology, Aarhus, Denmark, or from TAG Copenhagen, Copenhagen, Denmark. General purpose DNA sequencing was performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit With AmpliTaq DNA polymerase and an ABI PRISM 377 DNA sequencer. *Salmonella* genome sequence data were obtained from the following sources: sequence data for serovar Typhi strain CT18 were produced by the Microbial Genomes Sequencing Group at the Sanger Center. Serovar Paratyphi A ATCC 9510 and serovar Typhimurium LT2

SGSC1412 sequence data were produced by the Genome Sequencing Center, Washington University, St. Louis, Mo. During revision of this article, sequence data of serovar Typhi CT18 and serovar Typhimurium SGSC1412 were also published (27, 39). The following oligonucleotide primers based on serovar Typhi DNA sequences were used for screening by PCR and cloning of *chyA*-like sequences in *Salmonella*: stm1 (5'-CGCAGTTCTGAATGCGGAA-3'), stm3 (5'-TAATACCTGCTGTAGCAAGG-3'), sal1 (5'-CTCGTCAGCCCGGTAACGAC-3'), sal2 (5'-GAGGTAATAGGTAAGAATAC-3'), and sal6 (5'-CGGTA CCGATATCACCGATG-3'). The approximate annealing positions of these primers in the *chyA* locus are indicated below (see Fig. 2A). The primers sal1 and sal2 amplify a 1,101-bp DNA fragment that starts 73 bp upstream of the *chyA* start codon and ends 116 bp downstream of the *chyA* stop codon. The primers sal1 and sal6 amplify a 1,381-bp DNA fragment that starts 353 bp upstream of the *chyA* start codon and ends 116 bp downstream of the *chyA* stop codon. The primer stm1 anneals 170 bp downstream of the serovar Typhi *chyA* (*chyA*<sub>S<sub>Ty</sub></sub>) stop codon, and the primer stm3 anneals 1,568 bp upstream of the *chyA*<sub>S<sub>Ty</sub></sub> start codon.

**Plasmid and strain construction.** The relevant genotypes of plasmids used in this work are listed in Table 1. To construct a 900-bp in-frame deletion mutant of the *chyA* gene locus in serovar Typhi, we used a PCR-based strategy with DH5 $\alpha$  as the host strain. Serovar Typhi S2369/96 was used as the template. The primers p100 (5'-AGCAAATGACGAGCATGCGCTACGTGGATCCGATGA AAGAGAAC-3') and p101 (5'-TTGTTCTGCAAATATGTCGACCATAATC ATTTGCTT-3') amplify a 1,344-bp PCR fragment comprising the DNA from 1,320 bp upstream to 23 bp downstream of the *chyA*<sub>S<sub>Ty</sub></sub> start codon. This fragment contains a new restriction site for each of the enzymes *Bam*HI (sequence in boldface) and *Sph*I and *Sal*I (underlined sequences). The *Sal*I restriction site in this PCR fragment overlaps bp 4 to 9 in *chyA*<sub>S<sub>Ty</sub></sub>. The primers p102 (5'-CTTTT CGAGGTTCTGTGCGACTGATACATTTTCATT-3') and p103 (5'-GATGT CACTGAGGAGCTCATTGTCGGGATCCTCAACCCATTTG-3') amplify a 1,252-bp PCR fragment comprising the DNA from 21 bp upstream to 1,230 bp downstream of the *chyA*<sub>S<sub>Ty</sub></sub> stop codon. This fragment contains a new restriction site for each of the enzymes *Bam*HI (sequence in boldface) and *Sac*I and *Sal*I (underlined sequences). The *Sal*I restriction site in this PCR fragment overlaps bp 904 to 909 in *chyA*<sub>S<sub>Ty</sub></sub>. The PCR fragments were digested with *Sph*I-*Sal*I and *Sal*I-*Sac*I, respectively, and were simultaneously ligated into a *Sph*I-*Sac*I-digested pUC18 cloning vector. The resulting construct, designated pJON101, was used as the source for a 2.6-kb *Bam*HI fragment containing the *chyA*<sub>S<sub>Ty</sub></sub> in-frame deletion, which was subsequently ligated into a *Bam*HI-digested pKO3 suicide donor plasmid, resulting in the construct pJON103. Using pJON103, the *chyA*<sub>S<sub>Ty</sub></sub> in-frame deletion was introduced onto the chromosome of Ty21a, as previously described (24), to generate the strain JON42. That JON42 carried the  $\Delta$ *chyA* allele in the chromosome was confirmed by PCR analysis and DNA sequence determination (data not shown). For overexpression of *Salmonella* *ClyA* proteins, PCR-amplified DNA sequences were cloned directly into pGEM-T Easy using DH5 $\alpha$  as the host strain. To avoid enhanced expression from the *lac* promoter carried on the pGEM-T Easy cloning vector, we used only plasmid constructs in which the amplified PCR fragments had been ligated in the orientation opposite to that of the *lac* promoter. The constructs pMWK21 and pJON94 (*chyA*<sub>S<sub>Ty</sub></sub>) and pJON80 (serovar Paratyphi A *chyA* [*chyA*<sub>S<sub>Pa</sub></sub>]) contain the PCR fragments obtained by using the oligonucleotide primers sal1 and sal2 with the strains S2369/96, Ty21a, and S3068/99, respectively, as templates. The construct pJON97 (*chyA*<sub>S<sub>Ty</sub></sub>) contains the PCR fragment obtained by using the

oligonucleotides sal1 and sal6 with S2369/96 as the template. The strain YMZ16 (*clxA<sub>EC</sub>::kan*) was created by the use of an allelic-exchange system involving the plasmid pPM103, with temperature-sensitive replication properties. The plasmid clone pYMZ71 contains the *clxA<sub>EC</sub>* locus within a 1.3-kb *KpnI-SspI* restriction fragment from the *E. coli* K-12 chromosome. A 1.2-kb *BamHI* restriction fragment containing the kanamycin resistance gene from the plasmid pUC4K was ligated into *BglII*-digested pYMZ71, resulting in the construct pYMZ103, with the kanamycin cassette inserted 71 bp into the *clxA<sub>EC</sub>* coding sequence. A 3.4-kb *EcoRI-ScaI* restriction fragment from pYMZ103, containing the insertionally inactivated *clxA<sub>EC</sub>* locus, was subsequently ligated into *EcoRI-SmaI*-digested pPM103. The resulting temperature-sensitive replicon carrying the inactivated *clxA* locus (*clxA::kan*) was then introduced by transformation into BEU616, which has a *ClyA*-positive phenotype and is hemolytic on blood agar plates (50). Tetracycline-resistant transformants were selected at 30°C on LB agar plates containing tetracycline. *ClyA*-negative colonies were found after the temperature was raised to 42°C, and the colonies were plated on blood agar plates. An isolate designated YMZ16 was selected for further studies from among colonies that were sensitive to tetracycline, indicating loss of the plasmid. That YMZ16 carried the *clxA::kan* allele in the chromosome was confirmed by PCR analysis and by phage P1-mediated transduction experiments. The strain MC1061 was transduced with P1 grown on YMZ16, and transductants were isolated by selection for kanamycin resistance, resulting in the strain YMZ19.

**Determination of cytolytic activity.** Bacterium-mediated lysis of erythrocytes was scored by a clearance zone on blood agar plates incubated for 16 to 17 h at 37°C. As a convenient assay for cytolysis activity, we quantified the release of hemoglobin from erythrocytes essentially as described previously (43). The bacteria were grown on LB agar plates at 37°C for 16 to 17 h, and the cells were then harvested and suspended in 1× phosphate-buffered saline (PBS). Fifty microliters of a bacterial suspension in 1× PBS (final densities, 1 × 10<sup>9</sup>, 4 × 10<sup>8</sup>, and 2 × 10<sup>8</sup> bacterial cells ml<sup>-1</sup> as specified in the figure and table legends) was mixed with 50 μl of an erythrocyte suspension (final density, ~2 × 10<sup>9</sup> erythrocyte cells ml<sup>-1</sup> in 1× PBS) in a 96-well microtiter plate. The microtiter plate was incubated for 45 min at 37°C prior to determination of the release of hemoglobin at 545 nm as described previously (43). For osmotic protection, saccharides were added at a final concentration of 30 mM in the assay wells as described previously (34). Saccharides were assumed to have the following molecular diameters according to published data (44, 45): maltose, 1.0 nm; dextrin 20, 1.6 nm; and dextrin 15, 2.2 nm. In accordance with a previous study (2), the mean molecular diameters of raffinose and dextran 4 were assumed to be 1.2 to 1.4 nm and 3.0 to 3.5 nm, respectively, on the basis of published data (44).

**Subcellular fractionation of serovar Typhi cells and protein analysis.** For subcellular localization of *ClyA<sub>STY</sub>*, serovar Typhi cells were grown on LB agar plates at 37°C for 16 to 17 h and subsequently harvested in saline (final density, 3 × 10<sup>10</sup> bacterial cells ml<sup>-1</sup>). The cells were pelleted, and the culture supernatants were filtered through a 0.45-μm-pore-size filter (Millipore) and used as the extracellular fraction. Periplasmic proteins were isolated from the sediment by osmotic shock, essentially as described earlier (25). The pelleted bacteria were washed with 20 mM Tris-HCl (pH 8.0) and resuspended in 0.25 volume (compared to the starting volume of harvested bacteria) of a solution containing 20% (wt/vol) sucrose, 20 mM Tris-HCl (pH 8.0), and 1 mM Na-EDTA. The mixture was incubated for 10 min at room temperature. Subsequently, the bacteria were pelleted and resuspended in 1 volume of ice-cold H<sub>2</sub>O. After incubation on ice for 10 min, the cells were removed by centrifugation at 12,000 × *g*. The supernatant was used as the periplasmic protein extract. The cell pellet was then disrupted by sonication in 1 volume of 20 mM Tris-HCl (pH 8.0). The cell debris and unbroken cells were removed by centrifugation at 5,000 × *g* for 10 min at 4°C, and the supernatant was subsequently fractionated into the membrane and cytosolic fractions by centrifugation at 10,000 × *g* for 30 min at 4°C. The supernatant was used as the cytosolic fraction, and the sediment was used as the membrane fraction. Extracellular, periplasmic, and cytosolic proteins were precipitated by addition of ice-cold trichloroacetic acid (final concentration, 10%), pelleted by centrifugation at 12,000 × *g*, washed with acetone, and dried under vacuum. The total protein concentration in the different subcellular fractions was subsequently adjusted to approximately 0.6 mg ml<sup>-1</sup> by dissolving the fractions in 1× Laemmli lysis buffer. The protein concentrations in the fractions were determined using the bicinchoninic acid protein assay kit (Pierce) following the instructions of the manufacturer. For analysis of protein contents in bacterial strains, the strains were grown on LB agar plates at 37°C and harvested after 16 to 17 h of incubation. The bacteria were resuspended in saline, and the concentration was estimated by measuring the optical density at 600 nm using a spectrophotometer. The samples were then adjusted to 10<sup>10</sup> cells ml<sup>-1</sup> in 1× Laemmli lysis buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblotting were performed as previously described (20, 21). For

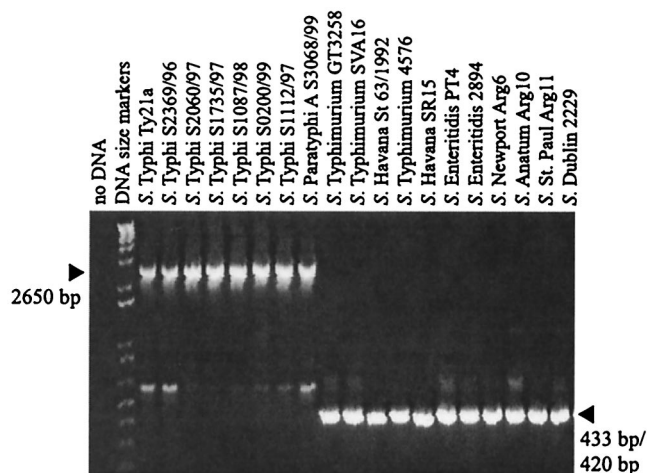


FIG. 1. PCR analysis of the *osmC clxA* DNA regions of various *Salmonella* isolates. PCR products were resolved by agarose gel electrophoresis following amplification using the primers *stm1* and *stm3*, which amplify a 433- or a 420-bp fragment in strains lacking both *clxA* and *osmC* and a 2,650-bp fragment in *clxA*<sup>+</sup> *osmC*<sup>+</sup> strains (arrowheads on right and left, respectively).

immunoblot analysis, we used a monoclonal antibody raised in mice against purified *ClyA<sub>EC</sub>* (S. N. Wai and B. E. Uhlin, unpublished data) or polyclonal antisera raised in rabbits against the purified proteins *ClyA<sub>EC</sub>* (34), *SlyA<sub>EC</sub>* (50), H-NS (19), β-lactamase (our unpublished data), and CRP (our unpublished data). The polyclonal *ClyA<sub>EC</sub>* and *SlyA<sub>EC</sub>* antisera were subtraction purified using a method that was described earlier (54). The monoclonal anti-*ClyA<sub>EC</sub>* antibody was used at a final dilution of 1:5,000 or 1:1,000 (as specified in the figure legends) with anti-mouse horseradish peroxidase-conjugated secondary antibody at a final dilution of 1:20,000. Polyclonal anti-*ClyA<sub>EC</sub>* and anti-*SlyA<sub>EC</sub>* antisera were used at final dilutions of 1:1,000 with horseradish peroxidase-conjugated anti-rabbit secondary antibody at a final dilution of 1:5,000. The polyclonal anti-H-NS, anti-CRP, and anti-β-lactamase antisera were used at a final dilution of 1:3,000 with horseradish peroxidase-conjugated anti-rabbit secondary antibody at a final dilution of 1:20,000. Immunoreactive bands were visualized using the ECL<sup>+</sup> chemiluminescence Western blotting detection system from Amersham Pharmacia Biotech, following the instructions of the manufacturer. Expression of catalase activity was evidenced by formation of bubbles on the addition of a few drops of 3% hydrogen peroxide to bacterial colonies on agar plates as described previously (51).

**Nucleotide sequence accession numbers.** Our nucleotide sequence data for the *clxA<sub>STY</sub>* locus in serovar Typhi S2369/96 and Ty21a and the *clxA<sub>SPA</sub>* locus in serovar Paratyphi A S3068/99 have been deposited in the EMBL database as entries AJ313032, AJ313033, and AJ313034.

## RESULTS

**Presence of *clxA*-homologues in human-specific *Salmonella* serovars.** In a previous study, no *clxA* gene could be detected in a clinical isolate of serovar Typhimurium by Southern hybridization (8). Similarly, by using PCR and oligonucleotide primers based on the *E. coli* K-12 sequence, *umu1* (5'-AATA TTTGTCGCTGC-3') and *p79* (5'-TGTC AACAGGTA ACTC TC-3'), or by performing Southern hybridization, we found that no *clxA*-like sequence could be amplified from or detected in the serovar Typhimurium LT2 strain GT3258 (our unpublished data). However, recent genome sequence analysis showed that there is a *clxA*-like sequence in the serovar Typhi strain CT18 but not in the serovar Typhimurium LT2 strain SGSC1412 (27, 39). To extend this analysis, we tested for the presence of a homologue to the *clxA<sub>EC</sub>* gene in various *Salmonella* genomes by means of PCR using the oligonucleotide

primers stm1 and stm3, which are based on the serovar Typhi genome sequence (Fig. 1). Our results clearly showed that *clyA*-like sequences are present in the genomes of all seven tested serovar Typhi isolates and in the tested serovar Paratyphi A isolate. On the other hand, all tested isolates of other serovars—three serovar Typhimurium isolates, two serovar Enteritidis isolates, two serovar Havana isolates, one serovar Anatum isolate, serovar Dublin, serovar Newport, and serovar St. Paul—were all assumed to lack the *clyA* locus, since a much shorter DNA fragment was obtained by PCR. A weaker band corresponding to ~500-bp fragments appeared in all cases and presumably represents partial unspecific base pairing of the oligonucleotide primers used.

DNA sequence analysis showed that there was 100% sequence identity between the serovar Typhi strains S2369/96 and CT18 over the 2,650-bp region between the primers stm1 and stm3, which encompasses the *clyA* locus. Furthermore, we found that the *clyA*-like open reading frame of serovar Typhi S2369/96 and CT18 shows 100% nucleotide sequence identity with the *clyA* coding sequence of the serovar Typhi vaccine strain Ty21a and 87% nucleotide sequence identity with *clyA*<sub>EC</sub>. Sequence analysis of the *clyA* homologue (*clyA*<sub>SPa</sub>) of serovar Paratyphi A S3068/99 revealed that this gene shows 98 and 86% nucleotide sequence identity with *clyA*<sub>STy</sub> and *clyA*<sub>EC</sub>, respectively, and 100% nucleotide sequence identity with the *clyA* locus of the serovar Paratyphi A strain ATCC 9510. In *E. coli* K-12, the *clyA* locus is located in close proximity and transcribed in the opposite direction to the *umuCD* operon, which is involved in resistance to UV-mediated stress (8). However, in typhoid *Salmonella*, other sequences were found in close proximity to the *clyA* locus (Fig. 2). In serovar Typhi, a 432-bp open reading frame shows 79% nucleotide sequence identity with the *E. coli* gene *osmC*, encoding an osmotically inducible protein. The *osmC* homologue starts 552 and ends 121 bp downstream of the *clyA*<sub>STy</sub> stop codon, i.e., it is transcribed in the direction opposite to that of *clyA*<sub>STy</sub>. A 636-bp open reading frame in the same direction as *clyA*<sub>STy</sub>, showing no significant similarity to that encoding any known protein according to computer searches, starts 766 and ends 131 bp upstream of the *clyA*<sub>STy</sub> sequence. Sequence comparisons showed the same arrangement of open reading frames around the *clyA* locus in serovar Paratyphi A as in serovar Typhi (Fig. 2). When we analyzed the shorter PCR fragments (433 bp) amplified from the serovar Typhimurium strains SVA16, 4576, and GT3258 obtained using the oligonucleotides stm1 and stm2, we found that both the *clyA* locus and the 636-bp open reading frame (see above) were absent in all the isolates. Al-

though these strains carry an *osmC*-like sequence, a 2,265-bp DNA region spanning from 1,282 bp upstream of what would correspond to the *clyA*<sub>STy</sub> start codon to 71 bp downstream of the *clyA*<sub>STy</sub> stop codon was found to be missing. We also noted the presence of a novel 48-bp DNA sequence positioned at the junction of this apparent 2,265-bp deletion (Fig. 2B). Computer searches, however, did not reveal any significant similarity that could suggest the origin of this 48-bp DNA sequence. DNA sequence analysis confirmed that there was 100% sequence identity over the region encompassed by the oligonucleotides stm1 and stm3 in SVA16, 4576, and GT3258. According to sequence analysis, the same region was absent in the other nontyphoid *S. enterica* serovars. The two serovar Havana isolates SR15 and St 63/1992 additionally lacked 13 bp in the 48-bp unique DNA fragment (Fig. 2B). Hence, the PCR product obtained from the serovar Havana strains using stm1 and stm3 was slightly smaller (420 bp), which was also supported by gel electrophoresis (Fig. 1). In conclusion, these findings expand the host range of ClyA to typhoid *Salmonella* and show that the *clyA* locus and a novel 636-bp open reading frame found directly upstream of *clyA*<sub>STy</sub> and *clyA*<sub>SPa</sub> are absent in many *Salmonella* serovars, including Typhimurium.

**Activity and secretion of ClyA cytotoxin in typhoid *Salmonella*.** Analogous to *clyA*<sub>EC</sub>, the *clyA*<sub>STy</sub> and *clyA*<sub>SPa</sub> genes were predicted to encode polypeptides of 303 amino acids (mass, ~34,000 Da) that show 91 and 90% amino acid identity to ClyA<sub>EC</sub>, respectively. To assess whether ClyA<sub>STy</sub> and ClyA<sub>SPa</sub>, similarly to ClyA<sub>EC</sub>, exhibit lytic activity, we amplified the *clyA*<sub>STy</sub> and *clyA*<sub>SPa</sub> gene loci by PCR using the oligonucleotides sal1 and sal2 with the strains S2369/96 and S3068/99, respectively, as templates. The resulting 1,101-bp PCR products were cloned into the expression vector pGEM-T Easy, resulting in the constructs pMWK21 (*clyA*<sub>STy</sub>) and pJON80 (*clyA*<sub>SPa</sub>). The *clyA*<sub>STy</sub> locus was also amplified as a 1,381-bp fragment by PCR using the primers sal1 and sal6 and cloned into pGEM-T Easy, resulting in the construct pJON97. When introduced by transformation into the *E. coli* strain YMZ19 (*clyA::kan*), pMWK21, pJON80, and pJON97 all induced a strong lytic phenotype when cultured on blood agar plates, indicating that *clyA*<sub>STy</sub> and *clyA*<sub>SPa</sub> have cytolytic activity per se (Table 2). We subsequently tested whether the ClyA<sub>STy</sub> and ClyA<sub>SPa</sub> proteins could also be recognized by a monoclonal antibody raised against ClyA<sub>EC</sub>, since they are very similar to the *E. coli* protein. As evidenced by Western immunoblot analysis, the anti-ClyA<sub>EC</sub> antibody could be used to monitor ClyA<sub>STy</sub> and ClyA<sub>SPa</sub> (Fig. 3A). The strains YMZ19/pMWK21 and YMZ19/pJON97 were found to express high levels of

FIG. 2. (A) Schematic map of the gene arrangement in a 4.5-kb region containing the *clyA* locus in serovar Typhi S2369/96, serovar Typhimurium LT2, serovar Typhi JON42, and *E. coli* K-12. The regions were aligned using the *clyA*<sub>STy</sub> gene sequence as a reference. Base pair coordinates are indicated relative to the arbitrarily chosen end point. Open reading frames are shown as shaded arrows, and the 48-bp unique sequence in serovar Typhimurium is shown as an open box. The 12 bp of the  $\Delta$ *clyA*<sub>STy</sub> allele in JON42 are shown with start and stop codons underlined. The approximate positions of recognition sequences for the restriction endonuclease *Hinc*II (H) and of sequences corresponding to the primers stm1, stm3, sal1, sal2, and sal6 (small arrows) are indicated. The extents of the *clyA*<sub>STy</sub> DNA regions cloned in the constructs pMWK21 and pJON97 are shown as solid bars. ORF, open reading frame. (B) Alignment of nucleotide sequences flanking the *osmC clyA* DNA region of serovar Typhi S2369/96, serovar Paratyphi A ATCC9510, and serovar Typhimurium LT2. Start and stop codons are shown in boldface and are underlined. The 13 bp of the 48-bp "inserted fragment" that were absent in the serovar Havana strains SR15 and St 63/1992 (see Results) are shown in boldface. Positions are indicated relative to the *clyA* start codon (+1). (C) Alignment of *clyA* upstream sequences of serovar Typhi S2369/96 and the *clyA* promoter region of *E. coli* K-12. Promoter elements [(-35) and (-10)], the transcriptional start point (+1), the Shine-Dalgarno sequence (SD), the translational start codon (ATG), and binding sites for the CRP and FNR regulatory proteins (CRP/FNR), described for the *clyA*<sub>EC</sub> locus (13, 25, 50), are indicated.



TABLE 2. Expression of phenotypical levels of ClyA as evidenced by lysis<sup>a</sup> of horse erythrocytes in agar

Strain	Relevant characteristics	Lysis activity
<i>E. coli</i>		
YMZ19/pGEM-T Easy	<i>clyA::kan</i> ; vector	-
YMZ19/pMWK21	<i>clyA::kan clyA<sub>STy</sub><sup>+</sup></i>	++
YMZ19/pJON97	<i>clyA::kan clyA<sub>STy</sub><sup>+</sup></i>	++
YMZ19/pJON80	<i>clyA::kan clyA<sub>SPa</sub><sup>+</sup></i>	++
MC1061/pYMZ80	<i>clyA<sup>+</sup> clyA<sub>EC</sub><sup>+</sup></i>	++
MC1061/pUC18	<i>clyA<sup>+</sup></i> ; vector	-
M182/pGEM-T Easy	<i>crp<sup>+</sup></i> ; vector	-
M182 <i>crp</i> /pGEM-T Easy	<i>crp</i> ; vector	-
M182/pJON97	<i>crp<sup>+</sup> clyA<sub>STy</sub><sup>+</sup></i>	++
M182 <i>crp</i> /pJON97	<i>crp clyA<sub>STy</sub><sup>+</sup></i>	++
Serovar Typhi		
Ty21a	<i>clyA<sub>STy</sub><sup>+</sup></i>	+
JON42	$\Delta$ <i>clyA</i>	-
JON42/pJON94	$\Delta$ <i>clyA clyA<sub>STy</sub><sup>+</sup></i>	++
JON42/pGEM-T Easy	$\Delta$ <i>clyA</i> ; vector	-
Ty21a/pGEM-T Easy	<i>clyA<sup>+</sup></i> ; vector	+
Ty21a/pMWK21	<i>clyA<sup>+</sup> clyA<sub>STy</sub><sup>+</sup></i>	++
Ty21a/pJON97	<i>clyA<sup>+</sup> clyA<sub>STy</sub><sup>+</sup></i>	++
Ty21a/pUC18	<i>clyA<sup>+</sup></i> ; vector	++
Ty21a/pMMkatF2	<i>clyA<sup>+</sup> rpoS<sub>EC</sub><sup>+</sup></i>	+
S2369/96	<i>clyA<sub>STy</sub><sup>+</sup></i>	-
S1112/97	<i>clyA<sub>STy</sub><sup>+</sup></i>	-
Serovar Paratyphi A S3068/99	<i>clyA<sub>SPa</sub><sup>+</sup></i>	-

<sup>a</sup> Lysis was scored on blood agar plates containing horse erythrocytes as follows: ++, large lysis zones around individual colonies; +, small lysis zones around individual colonies; -, no lysis.

ClyA<sub>STy</sub> protein, and YMZ19/pJON80 was found to express large amounts of ClyA<sub>SPa</sub> protein, in contrast to the vector control strain YMZ19/pGEM-T Easy, which did not.

To test whether ClyA<sub>STy</sub> could be expressed and secreted in serovar Typhi, we introduced the constructs pMWK21 and pJON97 into the serovar Typhi strain Ty21a by transformation. Similar to *E. coli* K-12, overexpression of *clyA<sub>STy</sub>* in serovar Typhi resulted in a strong hemolytic phenotype, as detected on blood agar plates (Table 2). As shown by Western immunoblotting, a significant amount of ClyA<sub>STy</sub> protein was produced by Ty21a/pJON97 and Ty21a/pMWK21 (Fig. 3A), and the lytic-plate phenotype suggested that ClyA<sub>STy</sub> reached the serovar Typhi cell surface. How ClyA is translocated out of the bacterial cell is currently unclear in *E. coli* as well as *Salmonella*. Like ClyA<sub>EC</sub>, the *Salmonella* homologues did not show a canonical signal sequence according to computer analysis.

We performed subcellular fractionation (see Materials and Methods) to localize ClyA<sub>STy</sub> in the strains Ty21a/pGEM-T Easy, Ty21a/pMWK21, and Ty21a/pJON97 (Fig. 3B). Large amounts of the ClyA<sub>STy</sub> protein were found in both the cytosolic and periplasmic fractions of Ty21a/pJON97 and Ty21a/pMWK21 (Fig. 3B, lanes 3 and 4, and data not shown). This is similar to what has been found previously with ClyA<sub>EC</sub> in *E. coli* strains (25). Also, in the case of the vector control strain Ty21a/pGEM-T Easy there was an immunoreactive band presumably representing a small amount of ClyA<sub>STy</sub> in the periplasmic fraction (Fig. 3B, lane 8). As ClyA was present in both cytosolic and periplasmic fractions, the possibility could not be excluded that a fraction of periplasm was mixed in with the cytosol during the preparation of the extracts. Hence, we used the periplasmic enzyme  $\beta$ -lactamase as a marker protein

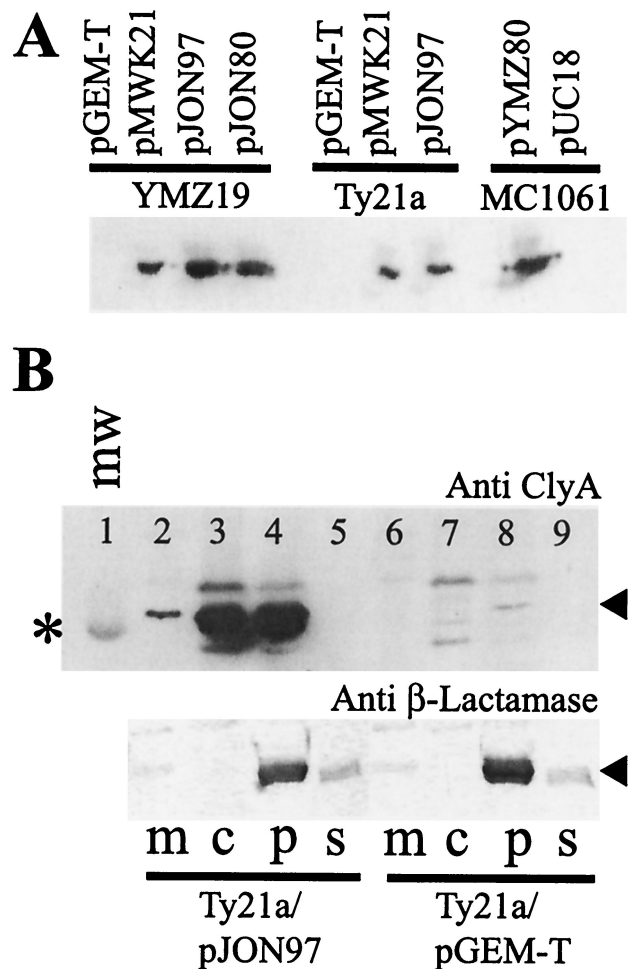


FIG. 3. Expression of cloned serovar Typhi, serovar Paratyphi A, and *E. coli* ClyA determinants and subcellular localization of ClyA<sub>STy</sub> in serovar Typhi. Immunoreactive bands were visualized using the ECL<sup>+</sup> blotting detection system (see Materials and Methods). (A) Immunoblot analysis, using a monoclonal ClyA<sub>EC</sub> antibody (final dilution, 1:5,000), of expression of cloned *clyA<sub>STy</sub>* (constructs pJON97 and pMWK21), *clyA<sub>SPa</sub>* (construct pJON80), and *clyA<sub>EC</sub>* (construct pYMZ80) gene loci in *E. coli* YMZ19 (*clyA::kan*) and MC1061 (*clyA<sup>+</sup>*) and serovar Typhi Ty21a. Approximately  $5 \times 10^7$  cells of each strain were used for the extracts applied on the gel. (B) Subcellular fractionation of serovar Typhi Ty21a/pJON97 (lanes 2 to 5) and Ty21a/pGEM-T Easy (lanes 6 to 9) in membrane (m), cytosolic (c), periplasmic (p), and supernatant (s) fractions. Above, immunoblot analysis using a monoclonal antibody raised against ClyA<sub>EC</sub> (final dilution 1:5,000) is shown. The position of the band corresponding to the 30-kDa protein of the molecular-mass marker (mw; lane 1) is indicated by an asterisk, and the position of the immunoreactive band representing the ClyA protein is indicated by an arrowhead. Below, immunoblot analysis of the same subcellular fractions (corresponding to lanes 2 to 9 in the upper blot) using a polyclonal antiserum against  $\beta$ -lactamase is shown. The position of the immunoreactive band representing  $\beta$ -lactamase is indicated by an arrowhead. An amount of the subcellular fraction equal to approximately 3  $\mu$ g of protein was applied in each lane of the gel.

and analyzed the same extracts using a polyclonal antiserum raised against  $\beta$ -lactamase. As shown in Fig. 3B, bottom, we found that the  $\beta$ -lactamase indeed was present in periplasmic fractions but not in cytosolic fractions, indicating that there was no or very minor contamination of the cytosol from the

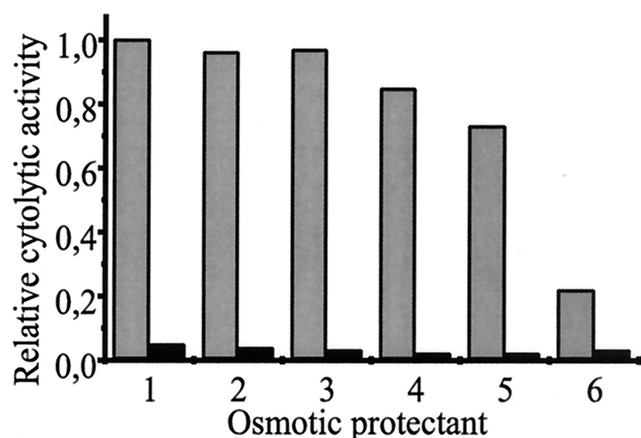


FIG. 4. Pore-forming activity of ClyA<sub>STy</sub> evidenced by osmotic protection using different sugar solutions in combination with suspensions of the serovar Typhimurium LT2 strains GT3258/pJON97 (*clxA<sub>STy</sub>*<sup>+</sup>) (shaded bars) and GT3258/pGEM-T Easy (vector control) (solid bars). Cytolytic activity was determined as described in Materials and Methods. The bacteria were grown on LB agar plates at 37°C for 16 to 17 h before being harvested. Approximately 10<sup>8</sup> bacterial cells were used for the samples applied in each well of the microtiter plate (final density, 10<sup>9</sup> bacterial cells ml<sup>-1</sup>). The activity of GT3258/pJON97 in 1× PBS was arbitrarily set to 1.0. Osmotic protectants (lane 1, 1× PBS; lane 2, maltose; lane 3, raffinose; lane 4, PEG1000; lane 5, PEG1500; and lane 6, dextran 4) were added at a final concentration of 30 mM.

periplasm. We also analyzed the same extracts using polyclonal antisera against the global regulatory proteins CRP and H-NS. As expected, these cytosolic proteins were mainly present in the cytosolic fractions, although there were also minor but detectable amounts of the proteins in the periplasmic fractions (data not shown). Taken together, these findings indicated that ClyA<sub>STy</sub> was efficiently translocated through the inner membrane in serovar Typhi.

To assess if, similarly to ClyA<sub>EC</sub>, the *Salmonella* cytotoxins act on erythrocytes by pore formation, we used an osmotic protection assay (see Materials and Methods). For this assay, it has been reported that the saccharide raffinose affords no protection against HlyA hemolysin- or ClyA<sub>EC</sub>-induced lysis whereas the larger molecule dextran 4 prevents lysis at 30 mM (2, 34). In hemolysis assays using a suspension of the serovar Typhimurium LT2 strain GT3258/pJON97 (*clxA<sub>STy</sub>*), lysis could be significantly reduced (approximately five times) in the presence of the osmoprotectant dextran 4 but not by the other, smaller saccharides (Fig. 4). Similar results were obtained with ClyA<sub>SPa</sub> as overexpressed in GT3258/pJON80 (data not shown). Based on these findings, we concluded that ClyA<sub>STy</sub> and ClyA<sub>SPa</sub>, like ClyA<sub>EC</sub>, form pores in erythrocyte membranes that are smaller than 3.0 to 3.5 nm (the molecular diameter of dextran 4) but larger than 2.2 nm (the molecular diameter of dextrin 15). To summarize, we found that the *clxA* loci of the human-specific typhoid *Salmonella* encode pore-forming proteins that can be released or secreted from the bacteria.

**ClyA<sub>STy</sub> is phenotypically expressed by serovar Typhi strain Ty21a.** The presence of a pore-forming determinant in the genomes of typhoid *Salmonella* prompted us to investigate whether these gene loci are phenotypically expressed or, as in *clxA<sub>EC</sub>*, are repressed and phenotypically silent. Interestingly,

upon cultivation on agar plates containing horse erythrocytes, the serovar Typhi strain Ty21a exhibited zones of hemolysis around the colonies while serovar Typhi S2369/96 and S1112/97 and serovar Paratyphi A S3068/99 did not (Table 2). To monitor the protein expression of *clxA<sub>STy</sub>* from the chromosome, the strains were subsequently harvested from the blood agar plates and analyzed by Western immunoblotting, using an enhanced detection system (Fig. 5A). Consistent with the phenotypes on blood agar, there was a detectable amount of ClyA<sub>STy</sub> protein accumulated in Ty21a, at a slightly higher level than in serovar Typhi S2369/96 and S1112/97, which could explain the difference in phenotype among the strains (Fig. 5A).

We noted that Ty21a, although able to lyse horse erythrocytes, did not exhibit lysis zones when grown on agar plates containing human or rabbit erythrocytes, and only a weak cytolytic activity was observed on blood agar containing sheep erythrocytes. Using cloned *clxA* genes from serovar Typhi and *E. coli*, we extended the analysis of the lysis of different erythrocytes by quantifying the release of hemoglobin by means of the assay described in Materials and Methods. *E. coli* DH5α cells containing various plasmid constructs were harvested and resuspended in 1× PBS and subsequently mixed with horse, sheep, human, goat, hen, and rabbit erythrocytes (Table 3). Evidently, both the ClyA<sub>STy</sub>- and ClyA<sub>EC</sub>-expressing cells showed the highest lytic activity towards horse erythrocytes, compared to, e.g., sheep and human erythrocytes, while the activity was very low against rabbit erythrocytes.

To confirm that the hemolytic phenotype of Ty21a was caused by expression of ClyA<sub>STy</sub>, we introduced by allelic replacement an in-frame deletion in the *clxA<sub>STy</sub>* gene locus on the chromosome of Ty21a (Fig. 2A) (see Materials and Methods). The resulting derivative, JON42 ( $\Delta$ *clxA<sub>STy</sub>*), was tested on blood agar plates containing horse erythrocytes and compared to the parent strain, Ty21a. As shown in Fig. 5B, the hemolytic phenotype was completely lost after the mutagenesis. Furthermore, our Western immunoblot analyses of these strains demonstrated that ClyA<sub>STy</sub> protein expression was abolished in JON42 (Fig. 5A). The hemolytic phenotype was restored by introduction of the plasmid pJON94, containing the *clxA* gene locus cloned from Ty21a, into JON42 (Table 2). Hence, we could conclude that the hemolytic phenotype of Ty21a was indeed caused by expression of the ClyA<sub>STy</sub> protein from the *clxA<sub>STy</sub>* locus.

**H-NS-mediated repression of ClyA<sub>STy</sub> production in *E. coli*.** Recently, the *clxA* promoter in *E. coli* K-12 has been characterized as an H-NS-silenced class I promoter that can be transcriptionally activated by CRP and/or FNR (50). An alignment of sequences 400 bp upstream of the *clxA* start codon revealed high (>90%) sequence identity between serovar Typhi and serovar Paratyphi A, but the *clxA* upstream regions were very different from that of *E. coli* K-12 (Fig. 2C). Sequence identity was observed only very close to the *clxA* start codon in *E. coli* K-12, overlapping with the Shine-Dalgarno sequence. We noted that a CRP/FNR DNA site seemingly is absent in the *clxA<sub>STy</sub>* and *clxA<sub>SPa</sub>* upstream sequences. Hence, a direct involvement of CRP and/or FNR as a modulator(s) of the expression of *clxA* in typhoid *Salmonella* appears less likely. To test whether there was a requirement for CRP for expression of *clxA<sub>STy</sub>* in *E. coli*, similar to what has been observed with *clxA<sub>EC</sub>* (50), we introduced the construct pJON97 into the *E.*

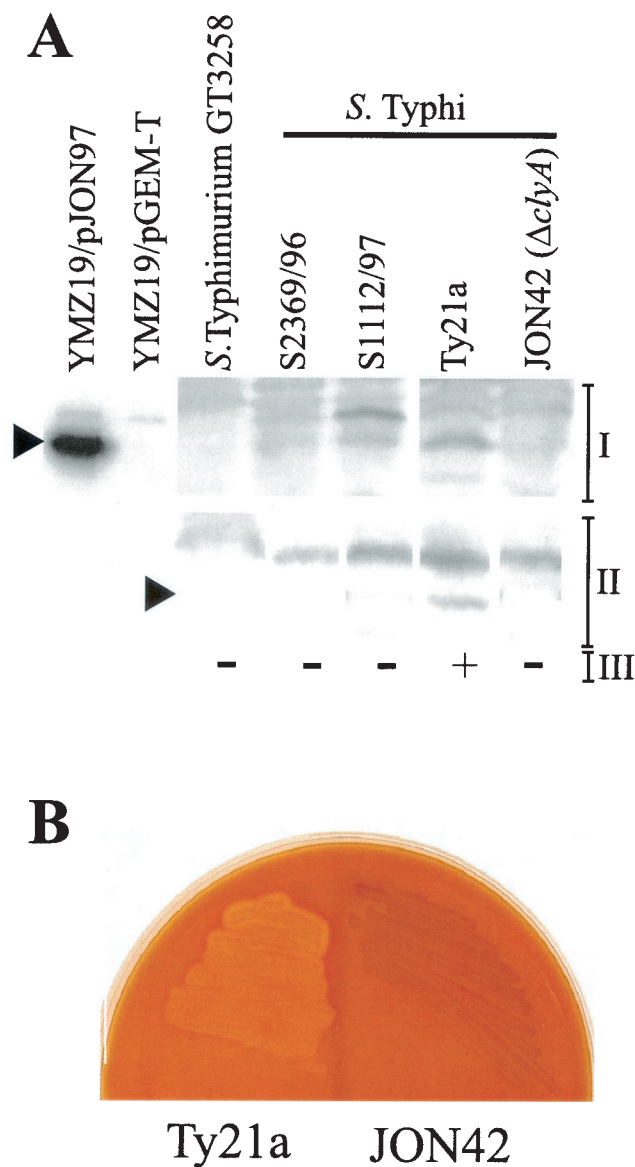


FIG. 5. Phenotypic expression of ClyA cytotoxin by serovar Typhi Ty21a and loss of lytic phenotype upon inactivation of the *clyA*<sub>STy</sub> gene locus. (A) Western immunoblotting detection of ClyA<sub>STy</sub> protein content in serovar Typhi strains using a polyclonal ClyA<sub>EC</sub> antiserum (I) or a monoclonal antibody raised against ClyA<sub>EC</sub> (final dilution, 1:1,000) (II) and the ECL<sup>+</sup> Western blotting detection system for visualization of immunoreactive bands (see Materials and Methods). The following approximate numbers of bacterial cells were used for the extracts loaded in the lanes. For the *Salmonella* strains,  $3 \times 10^8$  cells were used for the polyclonal antiserum and  $1 \times 10^8$  cells were used for the monoclonal antibody. For YMZ19/pJON97 (*clyA::kan clyA*<sub>STy</sub><sup>+</sup>) and YMZ19/pGEM-T Easy (*clyA::kan*; vector), approximately  $8 \times 10^6$  bacterial cells were used. The positions of reactive bands corresponding to ClyA are indicated with arrowheads on the left. The phenotypes of the strains on blood agar plates containing horse erythrocytes are indicated at the bottom (III) and were scored after 16 h of growth at 37°C (+, small lysis zones around individual colonies; -, no lysis). (B) Effect of inactivation of the *clyA*<sub>STy</sub> locus in serovar Typhi Ty21a. Shown are the phenotypes of Ty21a and the *clyA*<sub>STy</sub> in-frame deletion mutant JON42 on a blood agar plate containing horse erythrocytes after incubation at 37°C for 17 h.

TABLE 3. Lysis of various erythrocytes by ClyA<sub>STy</sub> and ClyA<sub>EC</sub>

Strain	Plasmid characteristics	Lysis (% of maximum) <sup>a</sup>					
		Horse	Sheep	Human	Goat	Hen	Rabbit
DH5α/pUC18	p(vector)	1.6	1.6	1.1	0.7	1.0	0.1
DH5α/pYMZ81	p(ClyA <sub>EC</sub> )	96	82	69	46	43	10
DH5α/pGEM-T Easy	p(vector)	2.5	0.9	0.6	0.9	2.7	0.1
DH5α/pJON97	p(ClyA <sub>STy</sub> )	57	37	35	30	21	0.5

<sup>a</sup> Lysis of erythrocytes was quantified using the hemolysis assay described in Materials and Methods. Shown are the percentage as of the maximal release of hemoglobin which was obtained by the addition of 1% Triton X-100 in the reaction wells. The bacteria were grown on LB agar plates at 37°C for 16 to 17 h before being harvested. Approximately  $2 \times 10^7$  bacterial cells were used for the samples applied in each assay well (final density,  $2 \times 10^8$  bacterial cells ml<sup>-1</sup>).

*coli* K-12 strains M182 and M182 *crp*. As indicated in Table 2, we found that there was no apparent difference in ClyA<sub>STy</sub> expression in these strains, indicating that there was no requirement for CRP.

The DNA of the upstream region (the 276 bp immediately upstream of the *clyA*<sub>STy</sub> start codon), like *clyA*<sub>EC</sub>, is rich in AT basepairs (65%), a hallmark of H-NS-regulated genes (1), and according to our data it may also be subject to silencing by H-NS. We used the constructs pJON97, containing the *clyA*<sub>STy</sub> structural gene and the DNA up to 353 bp upstream of the start codon, and pMWK21, containing the *clyA*<sub>STy</sub> locus, including a shorter part of the upstream region (73 bp). To measure the expression of cytolytic activity, we used bacterial suspensions and erythrocytes in a quantifying assay (see Ma-

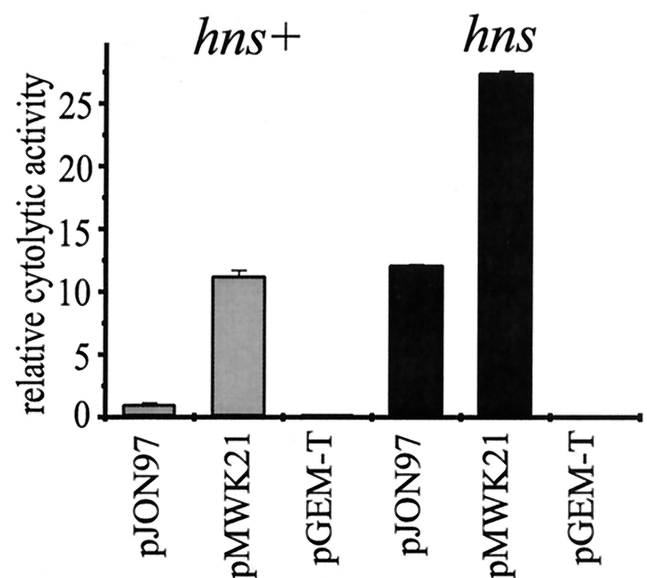


FIG. 6. H-NS-dependent repression of *clyA*<sub>STy</sub> in the *E. coli* K-12 strains YMZ19 (*clyA hns*<sup>+</sup>) (shaded bars) and YMZ16 (*clyA hns*) (solid bars) carrying different constructs. The bacteria were grown on LB agar plates at 37°C for 16 to 17 h before being harvested. Shown is the relative cytolytic activity as determined by quantification of the release of hemoglobin from erythrocytes (see Materials and Methods). Approximately  $4.0 \times 10^7$  bacterial cells were used for the samples applied in each well of the microtiter plate, giving a final density of  $4 \times 10^8$  cells per ml. The activity of YMZ19/pJON97 was arbitrarily set to 1.0. The error bars indicate standard errors of the mean from two separate experiments.



terials and Methods). As shown in Fig. 6, when introduced into the *E. coli* K-12 strains YMZ19 (*clyA*) and YMZ16 (*hns clyA*), the expression of cytolytic activity from pJON97 was substantially higher (more than ninefold) in the *hns* strain YMZ16 than in the *hns*<sup>+</sup> strain YMZ19. Derepression of *clyA*<sub>STy</sub> expression in the *hns* strain YMZ16 was also observed using the construct pMWK21, but the increase in cytolytic activity was less pronounced (approximately twofold). Hence, it appears that the *clyA*<sub>STy</sub> locus, similar to the *clyA* locus in *E. coli* K-12, may be subject to silencing by H-NS and that this feature may keep the gene in a repressed state. To investigate whether the enhanced expression of ClyA cytotoxin in Ty21a could be due to a reduced expression of H-NS in this strain, we performed Western immunoblotting using a polyclonal anti-H-NS antiserum. Our findings showed that H-NS was expressed at similar levels in all of the serovar Typhi strains Ty21a, S2369/96, and S1112/97 (data not shown).

In *E. coli* K-12 strains, the H-NS-mediated silencing of *clyA* expression has been shown to be relieved upon overexpression of the SlyA protein, possibly by competing with H-NS for binding at the *clyA* locus, although *clyA* expression does not require SlyA per se (25, 35, 50). Examination of available genomic data for serovar Typhi CT18 (39) revealed the presence of a 435-bp open reading frame showing 99 and 81% nucleotide sequence identity with *shyA* of serovar Typhimurium and *E. coli* K-12, respectively. Using an anti-SlyA<sub>EC</sub> antiserum, we performed immunoblot analyses of SlyA protein in serovar Typhi strains. The results showed that SlyA was expressed at a low level in all tested strains, i.e., Ty21a, S2369/96, and S1112/97, and the levels were similar to that observed in *E. coli* K-12 (data not shown). Since there was little or no difference among the serovar Typhi strains, we find it less likely that expression of SlyA was responsible for the increase in ClyA cytotoxin seen in Ty21a.

Serovar Typhi Ty21a has been shown to be defective for the alternative sigma factor  $\sigma^S$  (RpoS) (41). Since expression of many RpoS-dependent genes is modulated by global regulators, such as H-NS and CRP in *E. coli* (reference 16 and references therein), we tested whether the RpoS deficiency could be a possible reason for the hemolytic ClyA expression in Ty21a. To restore RpoS in Ty21a, we introduced the *rpoS*<sub>EC</sub><sup>+</sup> allele carried on the plasmid pMMkatF2 into Ty21a by transformation. As evidenced by the expression of catalase activity (see Materials and Methods), Ty21a/pMMkatF2 was considered to be *rpoS*<sup>+</sup> in contrast to the vector control strain Ty21a/pUC18. As indicated in Table 2, we found that the hemolytic phenotype of Ty21a was retained in this *rpoS*<sup>+</sup> background, suggesting that the *rpoS* mutation per se is not responsible for the enhanced expression of ClyA in Ty21a.

## DISCUSSION

Previous research with purified ClyA protein from *E. coli* K-12 demonstrated that ClyA is a pore-forming protein, with cytotoxic and apoptotic activities towards cultured mammalian cells (22, 25, 34, 47). As no experimental evidence of pore-forming factors in *Salmonella* had previously been presented, we aimed our experiments at elucidating features of the serovar Typhi and serovar Paratyphi A *clyA* homologues. Based on the strong similarity to *clyA*<sub>EC</sub>, both of the gene products (ClyA<sub>STy</sub> and ClyA<sub>SPa</sub>) were assumed to share features with

ClyA<sub>EC</sub>. To initially characterize these proteins, the *clyA*<sub>STy</sub> and *clyA*<sub>SPa</sub> determinants were cloned and overproduced from an expression vector. The proteins could be immunogenically recognized by a monoclonal antibody raised against ClyA<sub>EC</sub> and, analogous to ClyA<sub>EC</sub>, their molecular masses were determined to be ~34 kDa. The results from hemolysis assays using various saccharides as osmoprotectants demonstrated that ClyA<sub>STy</sub> and ClyA<sub>SPa</sub>, similar to ClyA<sub>EC</sub>, have a pore-forming activity towards erythrocytes. Hence, ClyA<sub>STy</sub> and ClyA<sub>SPa</sub> are the first examples of a pore-forming cytolysin encoded by *Salmonella*. It was earlier reported by Libby and coworkers (23) that they had occasionally observed weak zones of hemolysis around some colonies, especially in low-passage clinical isolates of *Salmonella*. However, it was not mentioned which *Salmonella* serovars were studied. In a cloning experiment using serovar Typhimurium 14028s, these authors obtained a clone containing a gene designated *shyA* (23). We and others subsequently showed that *shyA*, which also occurs in *E. coli*, encodes a regulatory protein that can activate *clyA* expression (26, 35). It remains to be elucidated whether the clinical isolates mentioned by Libby et al. (23) represent serovar Typhimurium, serovar Typhi, or other serovars.

The lytic phenotype of serovar Typhi Ty21a on horse erythrocytes, caused by natural expression of ClyA cytotoxin from the chromosomal gene locus, could not be detected on blood agar plates containing human or rabbit erythrocytes, and we observed only a weak cytolytic activity towards sheep erythrocytes. These findings were supported by our hemolysis assays, in which both ClyA<sub>STy</sub> and ClyA<sub>EC</sub> exhibited the strongest lysis activity against horse erythrocytes, while there was a lower activity against, e.g., sheep, human, and especially rabbit erythrocytes (Table 3). The reason for this difference in lysis of different erythrocytes is not known. In neither *Salmonella* nor *E. coli* is it established how ClyA is released from the bacterial cell. When overexpressed in *E. coli* strains, the ClyA<sub>EC</sub> protein was previously shown by subcellular localization to accumulate in periplasmic fractions (25). We obtained similar results upon subcellular fractionation of serovar Typhi Ty21a expressing ClyA<sub>STy</sub> naturally from the chromosomal gene locus or from an expression vector, indicating that the cytolysin was efficiently translocated across the inner membrane (Fig. 3B).

To our knowledge, Ty21a is the first reported phenotypically hemolytic *Salmonella* strain in which the genetic determinant has been identified. We observed that the serovar Typhi vaccine strain Ty21a lysed horse erythrocytes and had a hemolytic phenotype on blood agar plates (Fig. 5B). The lytic activity was abolished upon inactivation of the *clyA*<sub>STy</sub> gene locus in Ty21a and could be restored by the introduction of the *clyA*<sub>STy</sub> gene carried on a plasmid. We could therefore conclude that the hemolytic phenotype of Ty21a was caused by expression of ClyA cytotoxin. None of the other tested serovar Typhi isolates were detectably hemolytic on blood agar plates. It should be noted that the absence of hemolysis zones around colonies on blood agar does not necessarily mean that the *clyA* locus is silent. Recent findings using a sensitive system for Western immunoblot detection have demonstrated that, although non-hemolytic, some *E. coli* strains may contain a small amount of ClyA protein (36). Our present experiments, using the same system, demonstrated that there was a somewhat enhanced expression of ClyA<sub>STy</sub> from the chromosomal gene locus of Ty21a

compared to the nonhemolytic serovar Typhi isolates S2369/96 and S1112/97. Previous studies have shown that the threshold for appearance of hemolysis on blood agar plates is attained by relatively small increases in *clyA*<sub>EC</sub> expression (40), and thus the smaller quantities of ClyA<sub>STy</sub> produced by S2369/96 and S1112/97 could explain their nonhemolytic phenotypes.

At present, we do not know why Ty21a expresses a higher level of ClyA<sub>STy</sub> than other tested serovar Typhi isolates. Strain Ty21a has multiple mutations due to nitrosoguanidine-induced mutagenesis. The genetic alterations include mutations in genes affecting (i) Vi antigen biosynthesis, (ii) isoleucine-valine metabolism, (iii) H<sub>2</sub>S production, and (iv) growth rate (11, 12, 18). Although the *clyA*<sub>STy</sub> and *clyA*<sub>EC</sub> genes are highly homologous and both genes appear not to be fully derepressed, our sequence alignments showed that their promoter regions are very different (Fig. 2C). It appeared unlikely that CRP and/or FNR, as in *E. coli* K-12, is directly involved in the transcriptional activation of *clyA* expression in serovar Typhi or in serovar Paratyphi A, and no DNA site for these global regulators was identified upstream of the *clyA*<sub>STy</sub> and *clyA*<sub>SPa</sub> coding sequences. Experiments with the cloned *clyA*<sub>STy</sub> locus in *clyA* and *clyA* *hns* mutant *E. coli* K-12 strains demonstrated that *clyA*<sub>STy</sub>, similarly to *clyA*<sub>EC</sub>, may be subject to silencing by H-NS (Fig. 6). Our constructs used in these experiments contained the *clyA*<sub>STy</sub> locus with the 73 (pMWK21) or the 353 (pJON97) bp directly upstream of the *clyA*<sub>STy</sub> start codon, and our findings suggested that at least the 73 bp upstream of the *clyA* start codon are required for the regulation of *clyA* expression. This implies that *clyA*, analogous to the case in *E. coli*, may constitute a monocistronic operon in typhoid *Salmonella*. Even if *clyA*<sub>STy</sub> is a putative regulatory target for H-NS, the enhanced expression of the ClyA<sub>STy</sub> protein in Ty21a appeared not to be due to a reduced expression of H-NS in this strain, as evidenced by Western immunoblot analysis. Although our study showed that there was H-NS repression of *clyA*<sub>STy</sub> in *E. coli*, the results must be viewed with caution with respect to the situation in serovar Typhi. Further studies using, e.g., *hns* mutant serovar Typhi will hopefully reveal if H-NS plays a similar role in *Salmonella*.

Overproduction of the SlyA protein is known to activate ClyA expression in *E. coli* (25, 26, 35). As judged by our experimental determination of SlyA protein content, the phenotypic expression of ClyA cytotoxin in Ty21a is not likely to be caused by a higher level of SlyA<sub>STy</sub> in that strain. Although it has been suggested to contribute to the virulence of serovar Typhimurium (4), which lacks the *clyA* locus, the role of SlyA is not yet fully understood. Further research should reveal if SlyA plays a role in the activation of *clyA* expression in typhoid *Salmonella*.

In the present work, we establish that *clyA* is conserved in serovar Typhi and serovar Paratyphi A, while the gene locus is apparently absent in many other *Salmonella* serovars, including Typhimurium. Our PCR results and DNA sequencing showed that the coding regions are highly homologous among serovar Typhi (*clyA*<sub>STy</sub>), serovar Paratyphi A (*clyA*<sub>SPa</sub>), and *E. coli* (*clyA*<sub>EC</sub>). However, when the corresponding DNA regions in isolates of other *Salmonella* serovars were analyzed, a large apparent deletion (2,265 bp) was observed, removing the entire *clyA* locus and, interestingly, also a 636-bp open reading frame found directly upstream of *clyA*<sub>STy</sub> and *clyA*<sub>SPa</sub> (Fig. 2).

The *clyA* locus was replaced by a 48-bp DNA fragment that did not show any significant similarity to the sequences available in databases. Upon completion of the genome sequences of serovar Typhi CT18 (39) and serovar Typhimurium LT2 (27), many differences were found between the two serovars that may explain their different host specificities, e.g., several genes present in serovar Typhi are missing in serovar Typhimurium and vice versa. The absence of the *clyA* locus obviously reflects the fact that the *clyA* gene product is not correlated with virulence in some serovars. However, in the case of certain *Salmonella* serovars, we may consider that expression of ClyA cytotoxin could play a role at some point in particular types of infections. Hence, there could be selection for *clyA* in certain narrow-host-range serovars like Typhi and Paratyphi A that are pathogenic in humans only. Several lines of evidence support the idea that cytolytic factors are important for the pathogenesis of virulent bacteria (9), and the fact that typhoid *Salmonella* can express a protein with a pore-forming activity towards mammalian cells is intriguing. Our findings suggest that we must consider that ClyA may be a component of the arsenal of virulence factors possessed by typhoid *Salmonella*. The expression of ClyA by such strains may cause sublytic effects analogous to the host cell responses recently shown to be triggered by the  $\alpha$ -hemolysin from uropathogenic *E. coli* (45a). Whether this is the case with typhoid *Salmonella* and the vaccine strain Ty21a is an intriguing question, and the construction of a *clyA*<sub>STy</sub> mutant derivative (JON42) should allow the assessment of such aspects.

#### ACKNOWLEDGMENTS

We thank the Genome Sequencing Center, Washington University, for communication of DNA sequence data prior to publication. We thank Anna Aspán, Sven Bergström, Tina Broman, the Swedish Polar Research Secretariat, and the Center for Environmental Research for valuable assistance with sampling of *Salmonella* strains. We thank Hans Wolf-Watz for critical reading of the manuscript, Margareta Ramberg at SMI for valuable technical assistance, Dan Andersson for providing serovar Typhimurium LT2 GT3258, and Roland Rosqvist for providing serovar Dublin 2229.

This work was supported by grants from the Swedish Science Research Council (projects K2001-06X-10383-09C and B5101-1435) and the Swedish Foundation for International Cooperation in Research and Higher Education (STINT reference no. 99/966). S.N.W. was supported in part by a Visiting Scientist Fellowship from the Wenner-Gren Foundations. J.O. was supported by a Research Associate Fellowship and research funds from the Faculty of Medicine and Odontology, Umeå University.

#### REFERENCES

1. Atlung, T., and H. Ingmer. 1997. H-NS: a modulator of environmentally regulated gene expression. *Mol. Microbiol.* **24**:7-17.
2. Bhakdi, S., N. Mackman, J. M. Nicaud, and I. B. Holland. 1986. *Escherichia coli* hemolysin may damage target cell membranes by generating transmembrane pores. *Infect. Immun.* **52**:63-69.
3. Braun, V., and T. Focareta. 1991. Pore-forming bacterial protein hemolysins (cytolysins). *Crit. Rev. Microbiol.* **18**:115-158.
4. Buchmeier, N., S. Bossie, C. Y. Chen, F. C. Fang, D. G. Guiney, and S. J. Libby. 1997. SlyA, a transcriptional regulator of *Salmonella typhimurium*, is required for resistance to oxidative stress and is expressed in the intracellular environment of macrophages. *Infect. Immun.* **65**:3725-3730.
5. Casadaban, M. J., and S. N. Cohen. 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *J. Mol. Biol.* **138**:179-207.
6. Chen, L. M., K. Kaniga, and J. E. Galan. 1996. *Salmonella* spp. are cytotoxic for cultured macrophages. *Mol. Microbiol.* **21**:1101-1115.
7. Chopra, A. K., J. H. Huang, X. Xu, K. Burden, D. W. Niesel, M. W. Rosenbaum, V. L. Popov, and J. W. Peterson. 1999. Role of *Salmonella* enterotoxin in overall virulence of the organism. *Microb. Pathog.* **27**:155-171.
8. del Castillo, F. J., S. C. Leal, F. Moreno, and I. del Castillo. 1997. The

- Escherichia coli* K-12 *sheA* gene encodes a 34-kDa secreted hemolysin. Mol. Microbiol. **25**:107–115.
9. Finlay, B. B., and S. Falkow. 1997. Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev. **61**:136–169.
  10. Galan, J. E. 1996. Molecular genetic bases of *Salmonella* entry into host cells. Mol. Microbiol. **20**:263–271.
  11. Germanier, R., and E. Furer. 1983. Characteristics of the attenuated oral vaccine strain *S. typhi* Ty 21a. Dev. Biol. Stand. **53**:3–7.
  12. Germanier, R., and E. Furer. 1975. Isolation and characterization of Gal E mutant Ty 21a of *Salmonella typhi*: a candidate strain for a live, oral typhoid vaccine. J. Infect. Dis. **131**:553–558.
  13. Green, J., and M. L. Baldwin. 1997. The molecular basis for the differential regulation of the *hlyE*-encoded haemolysin of *Escherichia coli* by FNR and HlyX lies in the improved activating region 1 contact of HlyX. Microbiology **143**:3785–3793.
  14. Hacker, J., C. Hughes, H. Hof, and W. Goebel. 1983. Cloned hemolysin genes from *Escherichia coli* that cause urinary tract infection determine different levels of toxicity in mice. Infect. Immun. **42**:57–63.
  15. Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
  16. Hengge-Aronis, R. 1999. Interplay of global regulators and cell physiology in the general stress response of *Escherichia coli*. Curr. Opin. Microbiol. **2**:148–152.
  17. Hersh, D., D. M. Monack, M. R. Smith, N. Ghori, S. Falkow, and A. Zychlinsky. 1999. The *Salmonella* invasin SipB induces macrophage apoptosis by binding to caspase-1. Proc. Natl. Acad. Sci. USA **96**:2396–2401.
  18. Hone, D. M., S. R. Attridge, B. Forrest, R. Morona, D. Daniels, J. T. LaBrooy, R. C. Bartholomeusz, D. J. Shearman, and J. Hackett. 1988. A *galE* via (Vi antigen-negative) mutant of *Salmonella typhi* Ty2 retains virulence in humans. Infect. Immun. **56**:1326–1333.
  19. Johansson, J., and B. E. Uhlén. 1999. Differential protease-mediated turnover of H-NS and StpA revealed by a mutation altering protein stability and stationary-phase survival of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **96**:10776–10781.
  20. Johnston, K. H., and J. B. Zabriskie. 1986. Purification and partial characterization of the nephritis strain-associated protein from *Streptococcus pyogenes*, group A. J. Exp. Med. **163**:697–712.
  21. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680–685.
  22. Lai, X. H., I. Arencibia, A. Johansson, S. N. Wai, J. Oscarsson, S. Kalfas, K. G. Sundqvist, Y. Mizunoe, A. Sjöstedt, and B. E. Uhlén. 2000. Cytocidal and apoptotic effects of the ClyA protein from *Escherichia coli* on primary and cultured monocytes and macrophages. Infect. Immun. **68**:4363–4367.
  23. Libby, S. J., W. Goebel, A. Ludwig, N. Buchmeier, F. Bowe, F. C. Fang, D. G. Guiney, J. G. Songer, and F. Heffron. 1994. A cytolysin encoded by *Salmonella* is required for survival within macrophages. Proc. Natl. Acad. Sci. USA **91**:489–493.
  24. Link, A. J., D. Phillips, and G. M. Church. 1997. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. J. Bacteriol. **179**:6228–6237.
  25. Ludwig, A., S. Bauer, R. Benz, B. Bergmann, and W. Goebel. 1999. Analysis of the SlyA-controlled expression, subcellular localization and pore-forming activity of a 34 kDa haemolysin (ClyA) from *Escherichia coli* K-12. Mol. Microbiol. **31**:557–567.
  26. Ludwig, A., C. Tengel, S. Bauer, A. Bubert, R. Benz, H. J. Mollenkopf, and W. Goebel. 1995. SlyA, a regulatory protein from *Salmonella typhimurium*, induces a haemolytic and pore-forming protein in *Escherichia coli*. Mol. Gen. Genet. **249**:474–486.
  27. McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson. 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature **413**:852–856.
  28. Meacock, P. A., and R. N. Cohen. 1979. Genetic analysis of the interrelationship between plasmid replication and incompatibility. Mol. Gen. Genet. **174**:135–147.
  29. Monack, D. M., B. Raupach, A. E. Hromockyj, and S. Falkow. 1996. *Salmonella typhimurium* invasion induces apoptosis in infected macrophages. Proc. Natl. Acad. Sci. USA **93**:9833–9838.
  30. Mulvey, M. R., P. A. Sorby, B. L. Triggs-Raine, and P. C. Loewen. 1988. Cloning and physical characterization of *katE* and *katF* required for catalase HPII expression in *Escherichia coli*. Gene **73**:337–345.
  31. O'Brien, A. D., and R. K. Holmes. 1996. Protein toxins of *Escherichia coli* and *Salmonella*, p. 2788–2802. In F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, D.C.
  32. Ohl, M. E., and S. I. Miller. 2001. *Salmonella*: a model for bacterial pathogenesis. Annu. Rev. Med. **52**:259–274.
  33. Olsen, B., S. Bergström, D. J. McCafferty, M. Sellin, and G. Wiström. 1996. *Salmonella enteritidis* in Antarctica: zoonosis in man or humanosis in penguins? Lancet **348**:1319–1320.
  34. Oscarsson, J., Y. Mizunoe, L. Li, X. H. Lai, Å. Wieslander, and B. E. Uhlén. 1999. Molecular analysis of the cytolysin protein ClyA (SheA) from *Escherichia coli*. Mol. Microbiol. **32**:1226–1238.
  35. Oscarsson, J., Y. Mizunoe, B. E. Uhlén, and D. J. Haydon. 1996. Induction of haemolytic activity in *Escherichia coli* by the *slyA* gene product. Mol. Microbiol. **20**:191–199.
  36. Oscarsson, J., M. Westermark, L. Beutin, and B. E. Uhlén. 2002. The bacteriophage-associated Ehly1 and Ehly2 determinants from *Escherichia coli* O26:H– strains do not encode enterohemolysins *per se* but cause release of the ClyA cytolysin. Int. J. Med. Microbiol. **291**:625–631.
  37. Oscarsson, J., M. Westermark, S. Löfdahl, and B. E. Uhlén. 2000. Expression of a pore-forming cytotoxin (ClyA, HlyE, SheA) by typhoid *Salmonella*. Med. Microbiol. Immunol. **189**:44.
  38. Palmgren, H., D. McCafferty, A. Aspán, T. Broman, M. Sellin, R. Wollin, S. Bergström, and B. Olsen. 2000. *Salmonella* in sub-Antarctica: low heterogeneity in *Salmonella* serotypes in South Georgian seals and birds. Epidemiol. Infect. **125**:257–262.
  39. Parkhill, J., G. Dougan, K. D. James, N. R. Thomson, D. Pickard, J. Wain, C. Churcher, K. L. Mungall, S. D. Bentley, M. T. Holden, M. Sebaihia, S. Baker, D. Basham, K. Brooks, T. Chillingworth, P. Connor, A. Cronin, P. Davis, R. M. Davies, L. Dowd, N. White, J. Farrar, T. Felwell, N. Hamlin, A. Haque, T. T. Hien, S. Holroyd, K. Jagels, A. Krogh, T. S. Larsen, S. Leather, S. Moule, P. O'Gaora, C. Parry, M. Quail, K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S. Whitehead, and B. G. Barrell. 2001. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. Nature **413**:848–852.
  40. Ralph, E. T., J. R. Guest, and J. Green. 1998. Altering the anaerobic transcription factor FNR confers a hemolytic phenotype on *Escherichia coli* K12. Proc. Natl. Acad. Sci. USA **95**:10449–10452.
  41. Robbe-Saule, V., C. Coynault, and F. Norel. 1995. The live oral typhoid vaccine Ty21a is a *spoS* mutant and is susceptible to various environmental stresses. FEMS Microbiol. Lett. **126**:171–176.
  42. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
  43. Sansonetti, P. J., A. Ryter, P. Clerc, A. T. Maurelli, and J. Mounier. 1986. Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. Infect. Immun. **51**:461–469.
  44. Scherrer, R., and P. Gerhardt. 1971. Molecular sieving by the *Bacillus megaterium* cell wall and protoplast. J. Bacteriol. **107**:718–735.
  45. Schönherr, R., M. Hilger, S. Broer, R. Benz, and V. Braun. 1994. Interaction of *Serratia marcescens* hemolysin (ShlA) with artificial and erythrocyte membranes. Demonstration of the formation of aqueous multistate channels. Eur. J. Biochem. **223**:655–663.
  - 45a. Uhlén, P., A. Laestadius, T. Jahnukainen, T. Söderblom, F. Backhed, G. Celsi, H. Brismar, S. Normark, A. Aperia, and A. Richter-Dahlfors. 2000. Alpha-hemolysin of uropathogenic *E. coli* induces Ca<sup>2+</sup> oscillations in renal epithelial cells. Nature **405**:694–697.
  46. Vieira, J., and J. Messing. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
  47. Wallace, A. J., T. J. Stillman, A. Atkins, S. J. Jamieson, P. A. Bullough, J. Green, and P. J. Artymiuk. 2000. *E. coli* hemolysin E (HlyE, ClyA, SheA): X-ray crystal structure of the toxin and observation of membrane pores by electron microscopy. Cell **100**:265–276.
  48. Welch, R. A. 1991. Pore-forming cytolysins of gram-negative bacteria. Mol. Microbiol. **5**:521–528.
  49. Welch, R. A., C. Forestier, A. Lobo, S. Pellett, W. Thomas, Jr., and G. Rowe. 1992. The synthesis and function of the *Escherichia coli* hemolysin and related RTX exotoxins. FEMS Microbiol. Immunol. **5**:29–36.
  50. Westermark, M., J. Oscarsson, Y. Mizunoe, J. Urbonaviciene, and B. E. Uhlén. 2000. Silencing and activation of ClyA cytolysin expression in *Escherichia coli*. J. Bacteriol. **182**:6347–6357.
  51. Whittenbury, R. 1964. Hydrogen peroxide formation and catalase activity in the lactic acid bacteria. J. Gen. Microbiol. **35**:13–26.
  52. Willets, N. S., A. J. Clark, and B. Low. 1969. Genetic locations of certain mutations conferring recombination deficiency in *Escherichia coli*. J. Bacteriol. **97**:244–249.
  53. Williamson, C. M., G. D. Pullinger, and A. J. Lax. 1988. Identification of an essential virulence region on *Salmonella* plasmids. Microb. Pathog. **5**:469–473.
  54. Woods, J. P., J. F. Dempsey, T. H. Kawula, D. S. Barritt, and J. G. Cannon. 1989. Characterization of the neisserial lipid-modified azurin bearing the H.8 epitope. Mol. Microbiol. **3**:583–591.
  55. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.