THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 20, Issue of May 18, pp. 16660–16666, 2001
Printed in U.S.A.
© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.

In Vivo Proteolytic Degradation of the *Escherichia coli* Acyltransferase HlyC*

Received for publication, October 18, 2000, and in revised form, February 13, 2001
Published, JBC Papers in Press, February 15, 2001, DOI 10.1074/jbc.M009514200

Caterina Guzmán-Verri‡§¶, Esteban Chaves-Olarte‖, Fernando García‖, Staffan Arvidson‡, and Edgardo Moreno§

From the ‡Microbiology & Tumorbiology Center, Box 280, Karolinska Institute, S-171-77 Stockholm, Sweden, §Programa de Investigación en Enfermedades Tropicales, Escuela de Medicina Veterinaria, Universidad Nacional, Aptdo 304-3000 Heredia, Costa Rica, and ‖Centro de Investigación en Enfermedades Tropicales, Facultad de Microbiología, Universidad de Costa Rica, 1000, San José, Costa Rica

***Escherichia coli* hemolysin (HlyA) is the prototype toxin of a major family of exoproteins produced by Gram-negative bacteria known as "repeats in toxins." Only fatty acid-acylated HlyA molecules at residues Lys^{564} and Lys^{690} are able to damage the target cell membrane. Fatty acylation of pro-HlyA is dependent on the co-synthesized acyltransferase HlyC and the acylated form of acyl-carrier protein. By using a collection of *hlyA* and *hlyC* mutant strains, the processing of HlyC was investigated. HlyC was not detected by Western blot in an *E. coli* strain encoding *hlyC* and *hlyA*, but it was present in a strain encoding only *hlyC*. The *hlyC* mRNA pattern, however, was similar in both strains indicating that the turnover of HlyC does not occur at the transcriptional level. HlyC was detected in Western blots of cell lysates from an *E. coli* strain encoding HlyC and a HlyA derivative where both acylation sites were substituted. Similar results were obtained when HlyC was expressed in a *hlyA* mutant strain lacking part of a putative HlyC binding domain, indicating that this particular HlyA region affects HlyC stability. We did not detect HlyC in cell lysates from *hlyC* mutants with different abilities to acylate pro-HlyA, suggesting that the degradation of HlyC is not related to the HlyA acylation process. The protease systems ClpAP, ClpXP, and FtsH were found to be responsible for the HlyA-dependent processing of HlyC.**

Escherichia coli hemolysin (HlyA)[1] is the major representative of a family of proteins secreted by proteobacteria known as "repeats in toxin" (RTX). Other members of this family are leukotoxins from *Pasteurella hemolytica* and *Actinobacillus pleuropneumoniae*, the adenylate cyclase-hemolysin from *Bordetella pertussis*, the cytotoxin RtxA from *Vibrio cholerae*, and antigenically related exoproteins from *Neisseria meningitidis* and *Rhizobium leguminosarum* (1–12). Most of these proteins are pore-forming toxins proposed to play a role in the pathogenicity of these microorganisms. *E. coli* hemolysin (HlyA) is synthesized as an inactive precursor, which is activated through fatty acid acylation of residues Lys^{564} and Lys^{690} (13, 14) when both the HlyC protein and the acylated form of acyl-carrier protein are present (13, 15, 16).

HlyC is a small protein of 170 amino acid residues (19.8 kDa) homologous only to other known activator C proteins (17–20). Primary structure comparison of 13 activator C proteins from different bacterial species showed a high degree of similarity, suggesting a common function (17). At present, only HlyC from *E. coli* and CyaC from *B. pertussis* have been shown to be involved in the fatty acylation of their corresponding RTX toxin (13, 21). Recently, a novel type of fatty acyltransferase activity, that catalyzes the formation of an internal protein amide bond has been described for HlyC (22, 23), despite the fact that HlyC does not show any significant similarity to known acyl transferases (13, 19), the protein seems to acylate HlyA in an equimolar fashion, and it appears to be functionally consumed during the reaction (16, 22). In this respect, it is also notable that HlyC and HlyA are encoded in the same mRNA and therefore synthesized at nearly equimolar amounts (24, 25). The unique acylation reaction catalyzed by HlyC does not involve covalent attachment of fatty acid molecules to the acyltransferase but a direct interaction between acylACP-HlyC and pro-HlyA through a non-covalent ternary complex (22). A HlyC binding domain in pro-HlyA has been proposed (26, 27), and some highly conserved amino acid residues in the carboxyl and amino termini of HlyC essential for the acylation process have been identified (17, 28–30).

Understanding the mechanism of activation and processing of RTX systems is important for therapeutic applications and vaccine production. In this respect, promising results have been obtained using the *A. pleuropneumoniae* Apx toxins to elicit a protective immune response to prevent porcine pleuropneumonia (3). Here we present evidence that HlyC is degraded *in vivo* by different protease systems when HlyA is present.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Media—The recombinant plasmids (Table I) were propagated in *E. coli* 5K (Sm^r*lacYI tonA21 thr-1 supE44 thi* $r_k^-mk^+$) or XL1Blue (Stratagene, La Jolla, California). When two plasmids were introduced in the same host, their integrity was corroborated by restriction enzyme digestion. The protease system-deficient strains used are listed in Table I. Bacteria were grown in 2-fold YT broth or LB broth (31) supplemented with ampicillin (50 μg/ml) and/or chloramphenicol (10 μg/ml).

* This work was supported in part by Research Contract ICA4-CT-1999-10001 from the European Community, Research and Technological Development Project NOVELTARGETVACCINES, and Ministerio de Ciencia y Tecnología de Costa Rica/Consejo Nacional de Ciencia y Tecnología de Costa Rica, Costa Rica. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Recipient of a grant from the Swedish International Development Agency, as part of the Karolinska International Research Training Program. To whom correspondence should be addressed. Tel.: 506-2380761; Fax: 506-2381298; E-mail: piet@ns.medvet.una.ac.cr.

[1] The abbreviations used are: HlyA, *Escherichia coli* hemolysin; RTX, repeats in toxins; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; MALDI, matrix-assisted laser desorption/ionization; HlyC, *Escherichia coli* HlyC acyltransferase.

This paper is available on line at http://www.jbc.org

Downloaded from http://www.jbc.org/ by guest on October 1, 2020

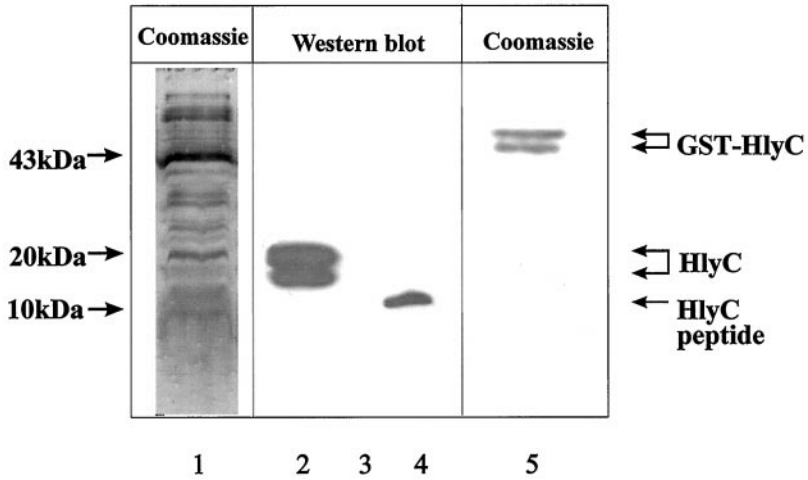

FIG. 1. **Electrophoretic analysis of HlyC in *E. coli* extracts.** *Lane 1*, 5KpFG1a Coomassie Blue-stained cell lysates containing HlyC; *lane 2*, Western blot of 5KpFG1a cell lysates using anti-HlyC antibodies; *lane 3*, Western blot of 5K(pANN202–812B,pFG1a) expressing *hlyA* and *hlyC* in *trans* in the absence of PMSF, using anti-HlyC antibodies; *lane 4*, same as *lane 3* in the presence of PMSF; *lane 5*, affinity-purified GST-HlyC that was Coomassie Blue-stained.

Construction of Recombinant DNA, Expression and Purification of GST-HlyC—HlyC was expressed as a GST tag fusion protein using vector pGEX-2T (Amersham Pharmacia Biotech, Uppsala, Sweden). Cloning and purification of the GST fusion protein was done according to the manufacturer's instructions (Amersham Pharmacia Biotech). Briefly, a PCR product from plasmid pFG1a encoding *hlyC* was obtained by using the following pair of primers: 5′-ACGTGAATTCTGAATATAAACAAACCATTAG-3′ and 5′-TATCGAATTCTTTAATTACCTCTTAACCAG-3′, containing *Eco*RI restriction site. Vector and PCR product were digested, ligated, and transformed into *E. coli* BL21-competent cells for propagation of the generated plasmid, pGEX2T-HlyC. GST-HlyC was functionally equivalent to wild type HlyC as judged by hemolytic activity assays. Expression of GST-HlyC was achieved by growing *E. coli* BL21 harboring pGEX2T-HlyC in 2-fold YT medium with aeration at 37 °C to mid exponential growth ($A_{600\ nm}$ = 0.5). Cultures were then induced with 0.1 mM IPTG at 30 °C for 2 h. Bacteria were collected by centrifugation at 7700 × *g*, resuspended in phosphate-buffered saline and lysed by sonication. After centrifugation at 12,000 × *g*, the supernatant was incubated with glutathione-Sepharose beads (Amersham Pharmacia Biotech) for 2 h at room temperature. Beads were washed twice with phosphate-buffered saline, and GST-HlyC was eluted with 10 mM glutathione (Sigma). The suspension was stored at −70 °C for further use.

Determination of Hemolytic Activity in Culture Supernatants—*E. coli* strains were grown in 20 ml of 2-fold YT medium containing the appropriate antibiotics at 37 °C with shaking. Samples were taken at late logarithmic phase of growth and analyzed as described (32). Culture supernatant from *E. coli* 5K(pANN202–812B, pUC18) was used as a negative control, and sheep erythrocytes lysed with water were used as total lysis control. The results were expressed as percentage of hemolysis produced by strain 5K(pANN202–812B, pFG1a) ("wild type hemolysin").

Antiserum Production and Purification—Rabbit anti-HlyC antibodies were produced by four intramuscularly applied boosts of GST-HlyC (250 μg) in complete (first boost) or incomplete (second to fourth boost) Freund adjuvant (Sigma). Antibodies from 2 ml of serum were adsorbed to GST-HlyC-Sepharose beads, eluted with 0.2 M glycine, pH 2.5, and collected in 1 M Tris, pH 9.0, according to Amersham Pharmacia Biotech recommendations. The antibodies were concentrated by ultrafiltration and stored at −20 °C in 50% glycerol.

Sample Preparation and Western Blotting—Cell samples from the various strains were taken at different time points throughout cell growth and resuspended in water, 0.1 mM PMSF (Sigma) unless otherwise stated. Cell lysates obtained by sonication were centrifuged at 100,000 × *g* for 1 h, and supernatants were collected. Twenty μg of total protein were separated on a 12.5% SDS-PAGE according to Ref. 33, transferred to a polyvinylidene difluoride membrane (Roche Molecular Biochemicals), and probed with the rabbit polyclonal antiserum against HlyC or with a rabbit polyclonal antiserum against HlyA provided by Dr. Ivaylo Gentschev (University of Würzburg, Würzburg, Germany). Probing with a peroxidase-labeled secondary antibody and developing were carried out using a chemiluminescence Western blotting kit (Roche Molecular Biochemicals). Samples of culture supernatants were prepared as described below.

Northern Blot Analysis—Total RNA from strains 5KpFG1a, 5K(pANN202–812B,pFG1a), and 5K(pACYC184,pUC18) at different time points was prepared using the RNeasy kit (Qiagen GmbH). For Northern blot analysis, 20 μg of total RNA were subjected to electrophoresis, transference to a Byodine A nylon membrane (Pall Ultrafine Filtration Corp.) and hybridization as described (34). A 450-base pair PCR product spanning nucleotides 726–1176 from Ref. 35, obtained from plasmid pFG1a was used as a probe to detect the 5′ end of *hlyC*. The fragment was radiolabeled to a specific activity of 1–5 × 10^8 cpm μg^{-1} with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) using a random primer labeling kit (Roche Molecular Biochemicals). After hybridization, the filters were washed once for 1 h at 42 °C in 2-fold 0.15 M NaCl plus 0.015 M sodium citrate containing 0.1% SDS. After 2 h of exposure, Northern blot images were processed using the ImageQuant software.

HlyC Immunoprecipitation—Cell samples from strains XL1BluepFG1a and XL1BluepUC18 were collected after IPTG induction according to Ref. 26. Seventy μg of total protein obtained after sonication of bacteria were mixed with 500 μl of immunoprecipitation buffer (150 mM NaCl, 1% Triton X-100, 10 mM Tris, pH 7.4, 1 mM EDTA, and 0.2 mM NaO_3V) as described (36). Twenty μg of protein A-Sepharose (Amersham Pharmacia Biotech) were added to the immunoprecipitation mix for preclearing. After 2 h at room temperature, the samples were centrifuged at 1600 × *g*. The supernatants were incubated with 1.5 μg of purified anti-HlyC antibody and incubated for 2 h at room temperature, followed by addition of 20 μl of protein A-Sepharose and further incubation for 2 h at room temperature. The samples were centrifuged at 1600 × *g* for 5 min, and the pellet was resuspended in 1 ml of immunoprecipitation buffer. After three washing steps, samples were resuspended in 50 μl of SDS-PAGE sample buffer for further analysis on a 12.5% SDS-PAGE and silver staining as described (37).

Analysis of HlyA by Two-dimensional Polyacrylamide Gel Electrophoresis—Supernatants corresponding to 5 × 10^8 cells at late logarithmic growth phase were precipitated with ice-cold trichloroacetic acid (final concentration 10%) for 1 h on ice. The pellet obtained after centrifugation was washed twice with ice-cold acetone and stored for no longer than 1 day at −20 °C. For analysis on two-dimensional polyacrylamide gel electrophoresis, the protocol described by Ludwig *et al.* (26) was followed with some modifications. Briefly, precipitated culture supernatants were resuspended in 20 μl of isoelectrofocusing sample buffer (62.5 mM Tris-HCl, pH 6.8, 0.5% SDS, and 10% glycerol) and 2 μl of 1 M Tris-HCl, pH 9.0. After heating at 56 °C for 15 min, 7.5 mg of urea/10 μl (ultra pure urea, Bio-Rad) were added, followed by two volumes of isoelectrofocusing lysis buffer: 9.5 M urea, 2% Nonidet P-40, 5% β-mercaptoethanol and 5% ampholines, pH range 3–10 (Bio-Lyte 3/10 ampholyte, Bio-Rad). The lower electrode solution was 13.6% H_3PO_4 and the upper electrode solution, 20 mM NaOH. After isoelectric focusing separation in a miniature two-dimensional electrophoresis cell, the proteins were separated in the second dimension by SDS-PAGE and stained with Coomassie Brilliant Blue as described (33, 37).

Site-directed Mutagenesis—Deletion of codons 10–18 in *hlyC* (pFG1a) was made by the ExSite PCR-based site-directed mutagenesis kit (Stratagene) with some modifications. Briefly, mutagenesis primers were designed according to Stratagene's recommendations and synthesized at Amersham Pharmacia Biotech. The sequence of the synthetic oligonucleotides is: 5′-AATCTCTAATGGTTTGTTTATATT and 5′-AGTTCTCCACTACACAGAAACT. One pmol of pFG1a prepared as described (31) was mixed with 15 pmol of each primer in 1% Me_2SO, 25 μmol of dNTP mix, and 2.5 units of *Taq* DNA polymerase premixed with 2.5 units of *Taq* extender PCR additive. The PCR was performed in a

Downloaded from http://www.jbc.org/ by guest on October 1, 2020

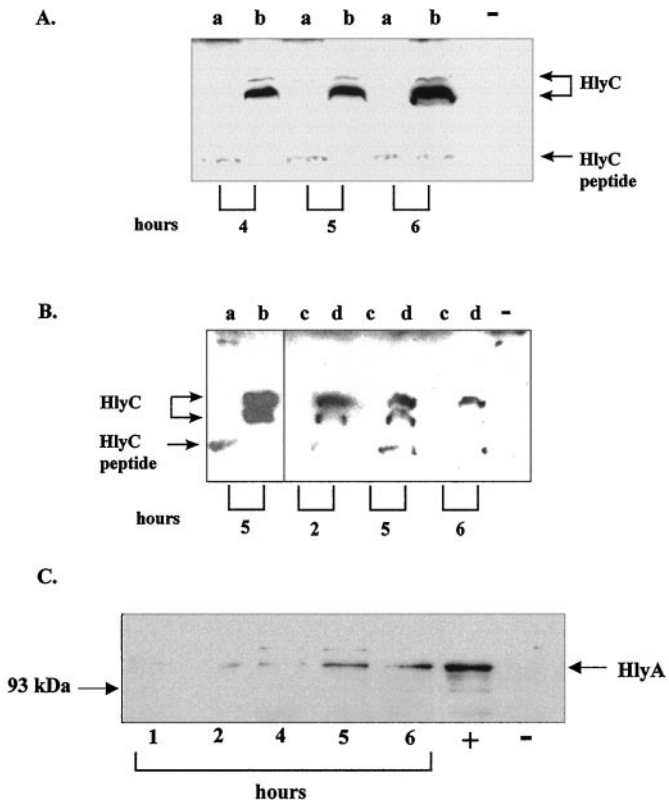

FIG. 2. **Expression of HlyC and HlyA throughout growth.** *A*, 20 μg of total protein from strain 5K(pANN202–812B,pFG1a) carrying *hlyABD* and *hlyC in trans* (*a*) or 5 μg of total protein from strain 5KpFG1a encoding only *hlyC* taken at different time points were analyzed by Western blot (*b*). *Lane* − contains a cell lysate from 5K(pANN202–812B,pUC18). *B*, Western blot analysis using anti-HlyC antibodies of a 5-h sample (20 μg) from strain 5K(pANN202–812) encoding *hly* in *cis* (*a*) is shown together with a positive control (*b*, 5KpFG1a cell lysate at 5 h). In the *c lanes,* cell lysates (20 μg) from strain 5K(pANN202–312*) taken at different time points and carrying *hly* in *cis* are shown, and in the *d lanes,* from strain 5KpFG1a encoding only *hlyC*. Samples from strain 5KpFG1a contain only 5 μg of total protein due to the high intensity signal. *C*, Western blot analysis of cell lysates taken at different time points from strain 5K(pANN202–812B,pFG1a) carrying *hlyABD* and *hlyC* in *trans* using anti-HlyA serum. *Lane* + contains a trichloroacetic acid-precipitated culture supernatant at 6 h from the same strain. *Lane* −, cell lysate from strain 5KpFG1a.

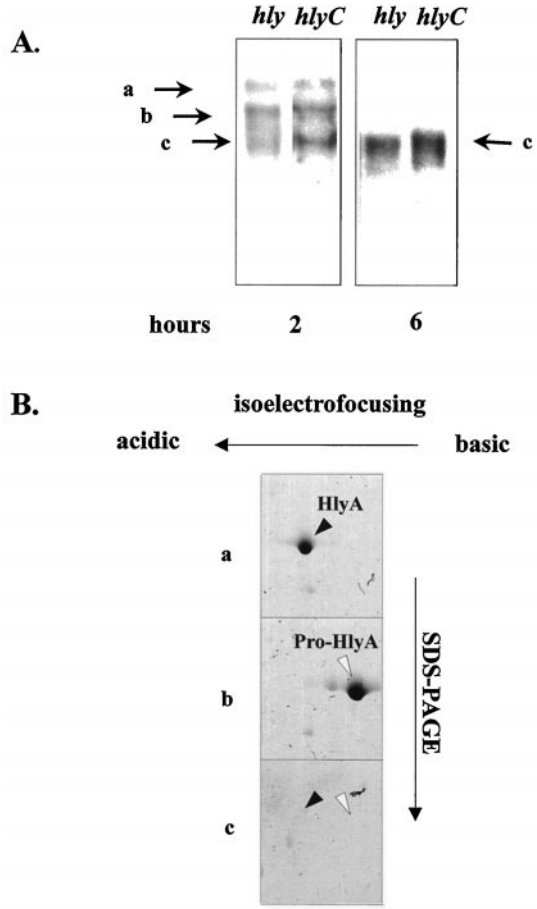

FIG. 3. **Northern blot analysis of *hlyC* mRNA and electrophoretic analysis of HlyA.** *A*, Northen blot analysis of *hlyC* transcripts (*a*, *b*, and *c*) at different time points from strains 5K(pANN202–812B,pFG1a) and 5KpFG1a. The size of the *hlyC* transcript from pFG1a is unknown due to the lack of a transcription stop in this construct. The same pattern of transcription was detected for both strains. No *hlyC* transcript was detected in total RNA extracted from control strain 5K(pACYC184,pUC18). *B*, two-dimensional polyacrylamide gel electrophoresis of culture supernatant from strain 5K(pANN202–812B,pFG1a) (*a*) showed a single protein spot that corresponds to fully acylated toxin according to control experiments: culture supernatant from the pro-HlyA producing strain 5KpANN202–812B (*b*) and culture supernatant from the non producing HlyA strain 5KpFG1a (*c*).

PerkinElmer Life Sciences GeneAmp PCR system 9600, and the cycles were as follows: one cycle at 94 °C for 4 min, 55 °C for 2 min, and 72 °C for 3 min; eight cycles at 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min, followed by 10 min at 72 °C. The digestion of the original template and polishing of the PCR product was performed according to the Stratagene protocol. After verifying the integrity of the PCR product by agarose gel electrophoresis, 5 μl of the mutagenesis reaction were ligated for 1 h at 37 °C with 4 units of T4 ligase and 5 μmol of ATP in a 12-μl final volume, and used to transform competent XL1Blue cells. Mutants were identified by terminator cycle sequencing using fluorescence-labeled dideoxynucleotides (ABI Prism dye terminator cycle sequencing core kit, PerkinElmer Life Sciences) and a pair of primers designed for that purpose. The sequencing primers corresponded to nucleotides 726–743 and 1030–1049 of the reference nucleotide sequence (35). The reactions were analyzed on an ABI Prism 373A DNA sequencer. Further transference of the mutated plasmid to *E. coli* 5K was performed by transformation according to Ref. 31.

Identification of Proteins by MALDI-Mass Spectrometry and Peptide Mass Mapping—Strains XL1BluepFG1a and XL1BluepUC18 were induced with IPTG as described (26). Cell lysates from both strains were obtained by resuspension in 2× SDS-PAGE sample buffer. Three hundred μg of total protein were precipitated with acetone for 20 min and resuspended in 60 μl of isoelectrofocusing lysis buffer. For two-dimensional separation, Immobiline DryStrip gels from Amersham Pharmacia Biotech were used and run according to manufacturer's instructions. After second dimension separation on SDS-PAGE and Coomassie staining, the proteins were localized by using reference spots from the same gel and from a Western blot run in parallel using the same sample. Identification of protein spots cut out from two-dimensional gels was carried out by MALDI-mass spectrometry and peptide mass mapping at the Protein Analysis Center, Karolinska Institute, Stockholm, Sweden. The tryptic peptide masses obtained were compared with the predicted masses of tryptic peptides from a protein sequence data base. All the analyzed protein spots generated peptides that matched the theoretical HlyC sequence.

RESULTS

HlyC Migrates as Two Protein Bands on SDS-PAGE—To raise a polyclonal antibody against HlyC, a GST-HlyC fusion protein was constructed, expressed, and purified. Affinity-purified GST-HlyC migrates on SDS-PAGE as two discrete bands corresponding to the expected molecular weight for the fusion protein (Fig. 1, *lane 5*). Affinity-purified antibodies against GST-HlyC were obtained using this preparation linked to Sepharose beads. Their specificity was evaluated by Western blot analysis of cell lysates from an *E. coli* strain encoding only *hlyC* and from a hemolytic strain carrying *hlyA* and *hlyC* in *trans*. Two bands close to 20 kDa were detected in cell lysates when HlyC was expressed alone (Fig. 1, *lanes 1* and *2*). These bands were not detected in cell lysates when HlyC was co-expressed with HlyA, and rather a 10-kDa protein band was observed (Fig. 1, *lane 4*). These results indicate that the produced

Downloaded from http://www.jbc.org/ by guest on October 1, 2020

TABLE I
Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Reference
E. coli strain		
SG22099	*clpA*319::mini-kan	(49)
SG22100	*clpB*::mini-kan	(50)
SG22101	*clpX*::kan	(49)
SG22166	*ftsH*1	(51)
BL21	*ompT*, *lon*	(52–54)
Plasmids		
pANN202–312*	pACYC184 with a 16.7-kb[a] *Sal*I fragment from plasmid pHly152 containing *hlyC*, *hlyA*, *hlyB*, *hlyD*, and the regulatory sequence *hlyR*	(26)
pANN202–812	pBR322 with a 16.7-kb *Sal*I fragment from plasmid pHly152 containing *hlyC*, *hlyA*, *hlyB*, *hlyD*, and the regulatory sequence *hlyR*	(55)
pAL26	pACYC184, *hlyR*, *hlyC*, *hlyA*, *hlyB*, *hlyD* from pHly152; ΔLeu^{722}–Gly^{724} in HlyA	(26)
pAL38	pACYC184, *hlyR*, *hlyC*, *hlyA*, *hlyB*, *hlyD* from pHly152; $Lys^{564} \rightarrow Ile$, $Lys^{690} \rightarrow Met$ in HlyA	(26)
pAL38B	pAL38; TT insertion in codon 141 of *hlyC*	(26)
pANN202–812B	pACYC184, *hlyR*, *hlyC*, *hlyA*, *hlyB*, *hlyD* from pHly152; TT insertion in codon 141 of *hlyC*	(56)
pGEX2T-*hlyC*	Glutathione *S*-transferase expression vector with a *tac* promoter, encoding GST-HlyC	This study
pFG1a	pUC18 with a 1.6-kb *Hinc*II fragment from pANN202–312 containing the *hly* promoter and *hlyC*	(26)
pΔ128–130	pFG1a, ΔGly^{128}–Val^{130} in HlyC	(17)
pG128V	pFG1a, $Gly^{128} \rightarrow Val$ in HlyC	(17)
pG128V, K129I	pFG1a, $Gly^{128} \rightarrow Val$, $Lys^{129} \rightarrow Ile$ in HlyC	(17)
pK129I	pFG1a, $Lys^{129} \rightarrow Ile$ in HlyC	(17)
pΔ10–18	pFG1a, ΔLeu^{10}–Ala^{18} in HlyC	This study

[a] kb, kilobase(s).

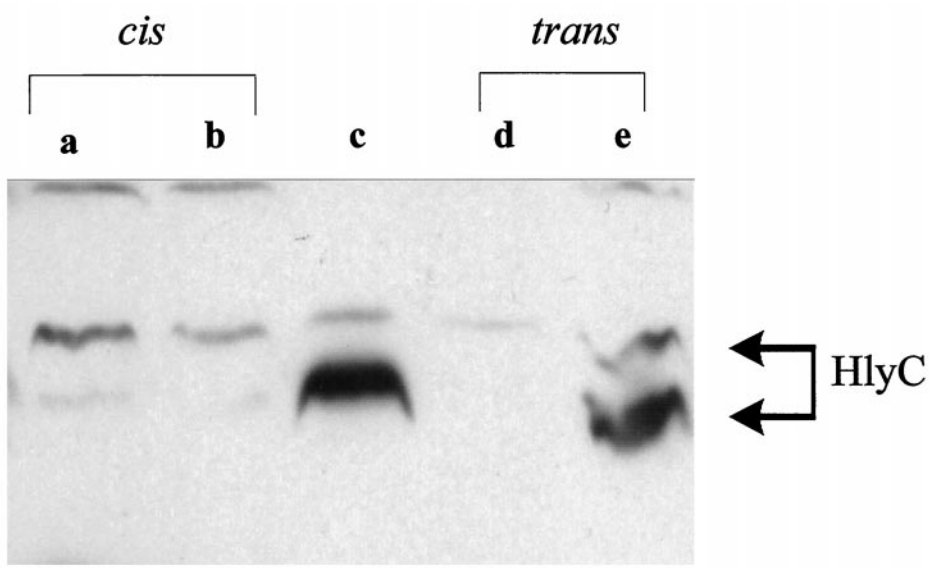

FIG. 4. **Western blot analysis using anti-HlyC antibodies of cell lysates from 5K strains expressing HlyC and different HlyA derivatives.** Cell lysate samples taken at late log growth phase from: *lane a*, strain 5K expressing a mutated form of HlyA ($HlyA_{\Delta 722-724}$) in *cis* to *hlyC* (20 μg); *lane b*, 5K expressing a mutated form of HlyA lacking both acylation sites in *cis* to *hlyC* (20 μg); *lane c*, 5KpFG1a expressing only *hlyC* (5 μg); *lane d,* 5K(pANN202–812B,pFG1a) encoding *hlyABD* and *hlyC* in *trans* (20 μg);. *lane e*, 5K expressing a mutated form of HlyA lacking both acylation sites in *trans* to *hlyC* (20 μg).

antibodies specifically recognize two forms of HlyC, in agreement with the two forms observed with purified GST-HlyC. In addition, reactivity of anti-HlyC antibodies with a lower molecular weight peptide in lysates where the full-length protein was not present, suggests proteolysis of the mature protein. This 10-kDa band was detected only when PMSF was added to the cell lysates (Fig. 1, *lanes 3* and *4*). The same molecular mass and mobility of the 20-kDa bands remained after treatment of lysates with and without β-mercaptoethanol, electrophoretic re-running of the isolated SDS-PAGE bands, and immunoprecipitation excluding degradation of the proteins by manipulation (data not shown).

The Presence of HlyA Correlates with HlyC Processing—To further investigate a possible role of HlyA in HlyC processing, cell samples from different 5K *E. coli* strains were taken throughout growth and analyzed by Western blot. HlyC was detected at all time points tested in *E. coli* carrying only *hlyC* (Fig. 2*A*). Instead, in lysates from the hemolytic strain encoding *hlyABD* and *hlyC* in *trans*, only the 10-kDa band was evident throughout growth. A small amount of the upper 20-kDa HlyC band was detected in the 5-h sample, indicating incomplete degradation at this particular time (Fig. 2*A*). When HlyA and HlyC were expressed in *cis*, again HlyC was not found, regardless of the copy number of the plasmids used (Fig. 2*B*). The presence of HlyA throughout growth was analyzed by Western blot using anti-HlyA antibodies (Fig. 2*C*). Increasing amounts of this protein were found in cell samples from *E. coli* carrying *hlyABD* and *hlyC* in *trans*, showing that HlyA was normally translated. These data indicate that the disappearance of HlyC proceeds throughout growth and is related to the presence of HlyA, indifferently of whether *hlyA* is encoded in *cis* or in *trans* to *hlyC* or in a high or low copy number plasmid. A small amount of the 10-kDa peptide band was detected in cell lysates obtained at late hours of growth from *E. coli* carrying only *hlyC*, suggesting that incomplete proteolysis may happen even in the absence of HlyA at later times.

To determine if the lack of HlyC in the *hlyCABD* encoding strain was due to impaired transcription of *hlyC,* total RNA was obtained throughout cell growth and analyzed by Northern blot using as probe the 5′ end sequence of *hlyC*. Equal amounts of *hlyC* RNA transcript were found at the time points tested in

Downloaded from http://www.jbc.org/ by guest on October 1, 2020

TABLE II
Features of the HlyC mutants analyzed

Change in HlyC	Detection of HlyC by Western blot in cells		HlyC in function of HlyA hemolytic activity[a]
	*hly*A$^-$	*hly*A$^+$	
No change	+	−	100
ΔGly^{128}–Val^{130}	+	−	0
Gly^{128} → Val	+	−	<1
Gly^{128} → Val, Lys^{129} → Ile	+	−	0
Lys^{129} → Ile	+	−	57
ΔLeu^{10}–Ala^{18}	−	−	<1

[a] Values are normalized relative to the hemolytic activity obtained with cotransformant 5K(pANN202–812B,pFG1a) and are representative of at least three independent assays.

cells carrying *hlyA* and *hlyC* in *trans* or in bacteria carrying only *hlyC* (Fig. 3*A*). Detection of three different *hlyC* encoding mRNA (*a*, *b*, and *c*) may be due to different transcription start points upstream from *hlyC* as has been described elsewhere (38, 39). An indirect proof of HlyC expression is the presence of acylated HlyA, since it is known that HlyA acylation is an HlyC-dependent reaction (13, 15, 16). The acylation status of HlyA was therefore assessed in culture supernatants from different strains by two-dimensional polyacrylamide gel electrophoresis. Fig. 3*B* (*panel a*) shows that a single protein spot corresponding to fully acylated toxin was detected in *E. coli* encoding *hlyABD* and *hlyC* in *trans*, whereas in a strain lacking *hlyC* (*panel b*) nonacylated HlyA was detected. This protein spot was not detected at all in *E. coli* encoding only HlyC (*panel c*). Thus, in *E. coli* carrying *hlyA* and *hlyC* in *trans*, HlyC must have been produced. HlyC was not co-secreted with HlyA to the extracellular medium as demonstrated by Western blot analysis of concentrated culture supernatants (data not shown). Altogether, these data indicate that HlyC is processed in *vivo* following expression of HlyA.

Acylation of HlyA Is Not Required for HlyC Processing—To confirm that the lack of HlyC is related to the presence of HlyA only and not to the toxin secretion machinery encoded by *hlyB* and *hlyD*, two *hlyA* mutant strains were used (Table I). When HlyC was co-expressed in *cis* or in *trans* to a derivative of HlyA lacking both acylation sites, HlyC was detected (Fig. 4, *lanes b* and *e*) as compared with control bacteria carrying wild type *hlyA* or only *hlyC* (Fig. 4, *lanes d* and *c*). Moreover, HlyC was detected in cell lysates from a strain encoding HlyC and another form of HlyA (HlyA$_{\Delta 722–724}$, Table I), lacking part of a putative HlyC binding domain (26, 27) (Fig. 4, *lane a*). Thus, HlyC processing is directly related to the presence of intact HlyA, since subtle changes in its sequence induce HlyC stabilization.

From previous work it is known that to modify HlyA, HlyC (i) binds to and then (ii) acylates HlyA (22). To examine whether HlyA acylation is a requisite for HlyC degradation, HlyC mutants with impaired abilities to activate pro-HlyA were used. Western blot analysis of cell samples from strains encoding different mutations in *hlyC* demonstrated that the HlyC mutants were produced at similar levels as wild type HlyC when expressed alone (Table II). Only a deletion at the amino-terminal region of the protein affected its stability. When the different mutated *hlyC* were expressed in *trans* to *hlyA*, none of the HlyC mutants was detected by Western blot. Determination of hemolytic activity of culture supernatants from these strains was used as assessment of HlyC function. As shown in Table II, these strains have different abilities to activate pro-HlyA. None of the mutated HlyC forms was detected when HlyA was present, regardless of their ability to acylate pro-HlyA. It is therefore concluded that HlyC processing is independent of the HlyA acylation reaction and probably depends solely on binding to HlyA.

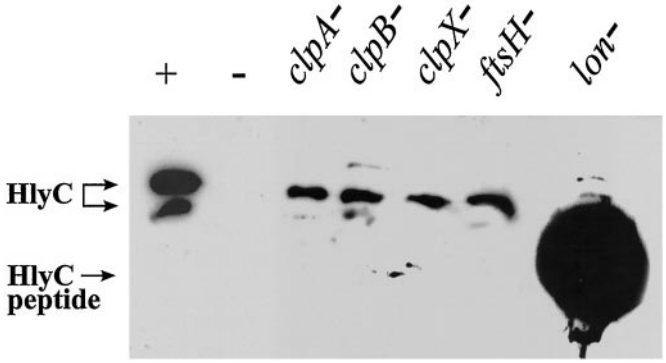

FIG. 5. **Immunoblot analysis of cell lysates from protease deficient strains transformed with plasmid pANN202–312*.** *Lane* + is a cell lysate (5 μg of total protein) from strain 5KpFG1a, encoding only *hlyC*, used as a positive control, and *lane* − is a cell lysate (20 μg of total protein) from strain 5KpANN202–312* encoding *hlyCABD* used as a HlyC negative control. The rest of the lanes contain 20 μg of total protein from the respective mutant strain.

HlyC Is Degraded by Different ATP-dependent Proteases—To determine if an *E. coli* protease system was involved in the processing of HlyC, a set of protease deficient strains transformed with a plasmid encoding the *hly* operon were tested by Western blot. The cell lysates taken at late logarithmic growth phase of Clp and FtsH mutants showed the two 20-kDa HlyC bands, without generating the 10-kDa product (Fig. 5). Thus, the Clp and FtsH protease systems are responsible of HlyC degradation. In contrast, only the 10-kDa product was detected in the *lon-ompT* mutant strain. This protein band was detected in large quantity as compared with the wild type situation (Fig. 5), suggesting that Lon and/or OmpT participate in additional degradation of this low molecular mass fragment. To determine if the 20-kDa bands detected in the protease deficient strains indeed correspond to HlyC, two-dimensional polyacrylamide gel electrophoresis in combination with Western blot were used. Interestingly, anti-HlyC antibodies recognized four HlyC spots in lysates from *E. coli clpB* carrying the *hly* determinant (Fig. 6*b*). The same protein pattern was observed in the control strain carrying only *hlyC*, indicating that the protein bands detected in the Clp and FtsH protease mutant strains are indeed HlyC (Fig. 6*a*). To further confirm this, the protein spots obtained from the control strain were cut out from a two-dimensional gel and sequenced. Each of the protein spots subjected to MALDI-mass spectrometry generated tryptic peptides with sequences corresponding to fragments of the HlyC protein, as indicated by peptide mass mapping. At present it is unknown what kind of modifications induce HlyC to migrate to more than one spot in two-dimensional gels.

DISCUSSION

The data presented here demonstrate that the HlyC bands and their degradation product were not the result of proteolysis during the separation procedures, but rather a physiological phenomenon related to the turnover of the HlyC protein. In general, HlyC has been estimated to be a single protein that participates in the acylation of HlyA hemolysin secreted by *E. coli* strains (23). Electrophoretic separation in combination with immunochemical detection, MALDI-mass spectrometry, and peptide mass mapping demonstrated that the four protein spots correspond to HlyC isoforms. Since the exact molecular size of each spot could not be determined by MALDI-mass spectrometry due to the low recovery of intact protein from a SDS gel, the molecular properties that generate the observed HlyC heterogeneity remain elusive. Based in our results and published data, it is possible to make some conjectures regarding the nature of the recorded differences. Stanley *et al.* (22)

Downloaded from http://www.jbc.org/ by guest on October 1, 2020

have shown that [^{3}H]palmitoyl-HlyC generated two bands in SDS-PAGE. Although distinctive acylation may explain the differences in charge, as is the case of HlyA (17, 26), the contribution in mass of the linked acyl groups is not enough to account for the differences in molecular weight observed among the HlyC spots. Alternative possibilities to explain the heterogeneity of HlyC are as follows. 1) The isoforms may be synthesized from mRNA transcripts of variable size, 2) normal translation of HlyC may be interrupted in a particular region within its sequence, or 3) HlyC might be susceptible to limited proteolysis even in the absence of HlyA. Although *hlyC* encoding mRNAs of various sizes have been described (this work and Refs. 38 and 39), it is unlikely that different mRNA transcripts would be responsible for the two HlyC isoforms detected, since expression of HlyC as a GST-fusion protein under the control of the *tac* promoter generated two isoforms as well. Furthermore, the two HlyC isoforms were present at all times tested, despite the fact that a long *hlyC* mRNA was detected at early hours of growth but not at late hours, in the wild type and control strain. Traces of the larger HlyC isoform were found in some cases, particularly at late hours of growth even when HlyA was expressed (Fig. 2*A*, *5 h lane a*; Fig. 4, *lane d*). This might be interpreted as the upper band being more stable than the lower band. The lower band could be the result of proteolysis that occurs independently of HlyA presence. Nevertheless, HlyC processing is enhanced when HlyA is expressed as demonstrated in our experiments.

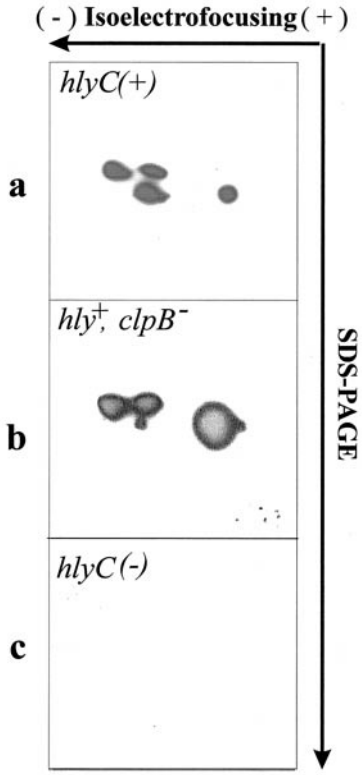

FIG. 6. **Analysis of HlyC by two-dimensional electrophoresis and immunoblot.** Cell samples from strain 5KpFG1a, encoding *hlyC* (*a*), and strain SG22100 (*clpB*), carrying the *hly* operon on plasmid pANN202–312* (*b*), were electrophoresed, blotted, and probed with anti-HlyC antibodies. A similar protein pattern was observed in both samples, despite the different absolute quantities among the spots. No HlyC spots were detected in cell samples from the control negative strain 5KpUC18 (*c*).

The similar levels of *hlyC* transcription, throughout growth of bacteria carrying only *hlyC* and bacteria carrying *hlyA* and *hlyC* in *trans*, show that the lack of HlyC when HlyA is present, was not due to impaired transcription of *hlyC*. Moreover, since *hlyA* is transcribed after *hlyC* to generate *hlyCA* or *hlyCABD* mRNA (24, 25), detection of HlyA is an indirect proof that *hlyC* is indeed transcribed. Fully acylated HlyA was detected in culture supernatants of cells carrying *hlyA* and *hlyC* in *trans* or in *cis* (26), demonstrating that HlyC must have been produced. Failure to detect HlyC regardless of whether *hlyC* was supplied in *cis* or in *trans* to *hlyABD* or in a high or low copy number plasmid indicates that this was not the result of transcriptional effects, differential stability of different *hlyC* containing mRNA, copy number, or *cis/trans* effects. We therefore conclude that HlyC is processed *in vivo* when HlyA is present.

The proteolytic processing of HlyC in the presence of HlyA described here is supported by the results of Hardie *et al.* (16). These investigators demonstrated that the function of HlyA, after reaching a saturation level, could only be re-established upon addition of HlyC, suggesting that the later protein is inactivated or consumed during the reaction. The degradation of HlyC appears to be dependent on the presence of HlyA and/or HlyB and HlyD, since the lack of HlyC was evident only when the entire *hly* determinant was expressed in *cis* or in *trans*. It is likely that the processing of HlyC is linked to the expression of HlyA rather than to the secretion of the toxin, as no particular interaction has been described between HlyC and the Hly secretion machinery. This view receives support from the fact that HlyC is detected in cell extracts of *E. coli* strains expressing mutated forms of HlyA but which secretion machinery remains intact.

A working model considering our results and those of Stanley *et al.* (22) is presented in Fig. 7. It has been suggested that, during the activation of HlyA, two consecutive events take place (22). First, there is interaction between acylACP-HlyC and pro-HlyA; second, the acylation of pro-HlyA proceeds. Our results suggest that, although the interaction event is important for HlyC processing, the acylation is not required, as

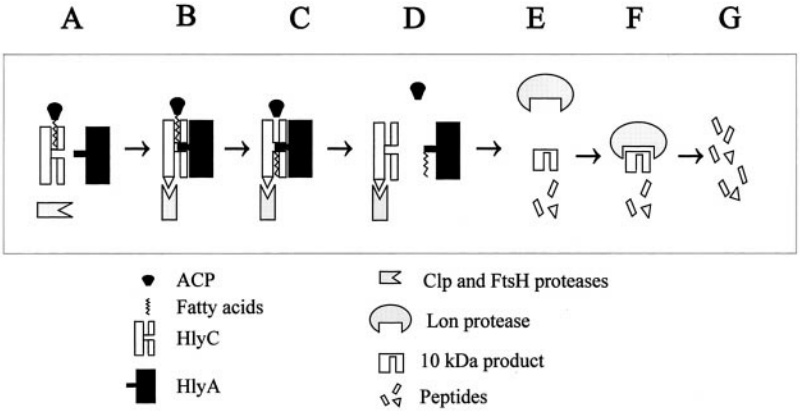

FIG. 7. **Model for HlyC degradation.** HlyC breakdown occurs when HlyC binds acyl-ACP in the presence of pro-HlyA (22) and the protease systems Clp and FtsH. *A*, *B*, binding of the binary complex acyl-HlyC-ACP to pro-HlyA induces a conformational change in HlyC, creating a recognition domain for interaction with proteases of the Clp and FtsH systems. The acylACP-HlyC-proHlyA ternary complex is generated. *C*, transference of the acyl chain from acyl-HlyC-ACP to pro-HlyA proceeds. *D*, the ternary complex is then dissociated. *E*, the Clp and FtsH protease systems perform the HlyC breakdown. *F* and *G*, further degradation of HlyC peptides is carried out by the Lon protease system.

Downloaded from http://www.jbc.org/ by guest on October 1, 2020

demonstrated by the lack of correlation between the hemolytic activity and the detection of HlyC (Table II). HlyC mutants, regardless of their ability to acylate or not pro-HlyA, are detected only when expressed out of the context of the *hly* determinant. This interpretation is also supported by the fact that a deletion of three amino acids in a putative HlyC binding site of HlyA (26) allows HlyC detection. The fact that HlyC was detected in a mutant where the acylation sites of HlyA were changed (26) might be due to the lack of the lysine residues *per se*, independently of its acylation status. Recently, Stanley *et al.* (22) proposed that HlyC generates a binary complex with acyl-ACP, before binding to pro-HlyA. HlyC is detected in the absence of HlyA, and under these conditions it has been shown that an acylated HlyC-ACP intermediate is formed, ruling out the possibility that this interaction results in HlyC processing. The interaction of HlyC-acyl-ACP complex with pro-HlyA should be the reason for HlyC degradation. Based on these results and our data, we postulate that a conformational change of HlyC occurs upon interaction with pro-HlyA, generating specific sites for different protease systems to process the active HlyC proteins. Considering that activation of one molecule of pro-HlyA seems to require one molecule of HlyC (16, 22, 23), it is likely that degradation of HlyC is necessary to prevent its intracellular accumulation as HlyA is continuously secreted throughout growth.

Protein processing is a well known mechanism of turnover in bacteria (40–42). The protease Clp systems, namely ClpXP and ClpAP, and the FtsH system seem to be responsible for the limited proteolysis of HlyC. The ATP-dependent protease Lon might be involved in further degradation of the 10-kDa polypeptide, since in the *lon* mutant strain this peptide band accumulated, as compared with the wild type strain. Furthermore, the 10-kDa band was detected only when PMSF is added during the preparation of the lysates, suggesting that the enzyme involved in the degradation of the 10-kDa product is a serine protease, as is the case of Lon. ClpB also seems to be involved in HlyC processing. This protein has been recently associated to the DnaK machinery (43), which is involved in the degradation of proteins such as the RpoH factors from *Bradyrhizobium japonicum* (44). Redundancy in protein degradation has been described in other cases like the cell division inhibitor SulA, the positive regulator of capsule transcription RcsA, Xis of phage λ, the heat shock sigma factor RpoH or σ^{32}, and SsrA-tagged proteins (45–48). Overlapping specificity of the different proteases may provide an effective means to accelerate the degradation of critical common substrates (47, 48).

Acknowledgments—We thank Dr. Werner Goebel's laboratory for providing wild type and HlyA mutants and Dr. Susan Gottesman for providing the protease mutants. We are grateful to Cristine Jacobs for stimulating discussions and Andrea Parra for technical help.

REFERENCES

1. Chang, Y. F., Ma, D. P., Shi, J., and Chengappa, M. M. (1993) *Infect. Immun.* **61,** 2089–2095
2. Chang, Y. F., Young, R., and Struck, D. K. (1989) *DNA* **8,** 635–647
3. Frey, J. (1995) *Trends Microbiol.* **3,** 257–261
4. Glaser, P., Sakamoto, H., Bellalou, J., Ullmann, A., and Danchin, A. (1988) *EMBO J.* **7,** 3997–4004
5. Jansen, R., Briaire, J., Smithe, H. E., Dom, P., Haesebrouck, F., Kamp, E. M., Gielkins, A. L., and Smits, M. A. (1995) *Infect. Immun.* **63,** 27–37
6. Koronakis, V., Cross, M., Senior, B., Koronakis, E., and Hughes, C. (1987) *J. Bacteriol.* **169,** 1509–1515
7. Kuhnert, P., Heyberger-Meyer, B., Nicolet, J., and Frey, J. (2000) *Infect. Immun.* **68,** 6–12
8. Lin, W., Fullner, K. J., Clayton, R., Sexton, J. A., Rogers, M. B., Calia, K. E., Calderwood, S. B., Fraser, C., and Mekalanos, J. J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 1071–1076
9. Sutton, J. M., Lea, E. J., and Downie, J. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 9990–9994
10. Thompson, S. A., Wang, L. L., West, A., and Sparling, P. F. (1993) *J. Bacteriol.* **175,** 811–818
11. Thompson, S. A., and Sparling, P. F. (1993) *Infect. Immun.* **61,** 2906–2911
12. Welch, R. A., Dellinger, E. P., Minshew, B., and Falkow, S. (1981) *Nature* **294,** 665–667
13. Issartel, J. P., Koronakis, V., and Hughes, C. (1991) *Nature* **351,** 759–761
14. Stanley, P., Packman, L. C., Koronakis, V., and Hughes, C. (1994) *Science* **266,** 1992–1996
15. Goebel, W., and Hedgpeth, J. (1982) *J. Bacteriol.* **151,** 1290–1298
16. Hardie, K. R., Issartel, J. P., Koronakis, E., Hughes, C., and Koronakis, V. (1991) *Mol. Microbiol.* **5,** 1669–1679
17. Guzmán-Verri, C., Garcia, F., and Arvidson, S. (1997) *J. Bacteriol.* **179,** 5959–5962
18. Hartlein, M., Schiell, S., Wagner, W., Rdest, U., Kreft, J., and Goebel, W. (1983) *J. Cell. Biochem.* **22,** 87–97
19. Hughes, C., Issartel, J. P., Hardie, K., Stanley, P., Koronakis, E., and Koronakis, V. (1992) *FEMS Microbiol. Immunol.* **105,** 37–44
20. Nicaud, J. M., Mackman, N., Gray, L., and Holland, I. B. (1985) *FEBS Lett.* **187,** 339–344
21. Hackett, M., Guo, L., Shabanowitz, J., Hunt, D. F., and Hewlett, E. L. (1994) *Science* **266,** 433–435
22. Stanley, P., Hyland, C., Koronakis, V., and Hughes, C. (1999) *Mol. Microbiol.* **34,** 887–901
23. Trent, M. S., Worsham, L. M., and Ernst-Fonberg, M. L. (1998) *Biochemistry* **37,** 4644–4652
24. Koronakis, V., Cross, M., and Hughes, C. (1988) *Nucleic Acids Res.* **16,** 4789–4800
25. Welch, R. A., and Pellet, S. (1988) *J. Bacteriol.* **170,** 1622–1630
26. Ludwig, A., García, F., Bauer, S., Jarchau, T., Benz, R., Hoppe, J., and Goebel, W. (1996) *J. Bacteriol.* **178,** 5422–5430
27. Stanley, P., Koronakis, V., Hardie, K., and Hughes, C. (1996) *Mol. Microbiol.* **20,** 813–822
28. Trent, M. S., Worsham, L. M., and Ernst-Fonberg, M. L. (1999) *Biochemistry* **38,** 9541–9548
29. Trent, M. S., Worsham, L. M., and Ernst-Fonberg, M. L. (1999) *Biochemistry* **38,** 8831–8838
30. Trent, M. S., Worsham, L. M., and Ernst-Fonberg, M. L. (1999) *Biochemistry* **38,** 3433–3439
31. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
32. Bauer, M. E., and Welch, R. A. (1996) *Infect. Immun.* **64,** 167–175
33. Laemmli, U. K. (1970) *Nature* **227,** 680–685
34. Janzon, L., Lofdahl, S., and Arvidson, S. (1986) *FEMS Microbiol. Lett.* **33,** 193–198
35. Hess, J., Wels, W., Vogel, M., and Goebel, W. (1986) *FEMS Microbiol. Lett.* **34,** 1–11
36. Harlow, E., and Lane, D. (1999) *Using Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
37. Coligan, J. E., Dunn, B. M., Ploegh, H. L., Speicher, D. W., and Wingfield, P. T. (1995) in *Current Protocols in Protein Science* (Benson Chanda, V., ed) p. 10.5.1, John Wiley & Sons, Inc., New York
38. Koronakis, V., and Hughes, C. (1988) *Mol. Gen. Genet.* **213,** 99–104
39. Koronakis, V., Cross, M., and Hughes, C. (1989) *Mol. Microbiol.* **3,** 1397–1404
40. Gottesman, S., Maurizi, M. R., and Wickner, S. (1997) *Cell* **91,** 435–438
41. Gottesman, S. (1999) *Curr. Opin. Microbiol.* **2,** 142–147
42. Porankiewicz, J., Wang, J., and Clarke, A. K. (1999) *Mol. Microbiol.* **32,** 449–458
43. Zolkiewski, M. (1999) *J. Biol. Chem.* **274,** 28083–28086
44. Urech, C., Koby, S., Oppenheim, A. B., Münchbach, M., Hennecke, H., and Narberhaus, F. (2000) *Eur. J. Biochem.* **267,** 4831–4839
45. Kanemori, M., Nishihara, K., Yanegi, H., and Yura, T. (1997) *J. Bacteriol.* **179,** 7219–7225
46. Leffers, G. G., Jr., and Gottesman, S. (1998) *J. Bacteriol.* **180,** 1573–1577
47. Kanemori, M., Yanagi, H., and Yura, T. (1999) *J. Bacteriol.* **181,** 3674–3680
48. Wu, W.-F., Zhou, Y., and Gottesman, S. (1999) *J. Bacteriol.* **181,** 3681–3687
49. Gottesman, S., Clark, W. P., de Crecy-Lagard, V., and Maurizi, M. R. (1993) *J. Biol. Chem.* **268,** 22618–22626
50. Squires, C., and Squires, C. L. (1992) *J. Bacteriol.* **174,** 1081–1085
51. Gottesman, S., Roche, E., Zhou, Y., and Sauer, R. T. (1998) *Genes Dev.* **12,** 1338–1347
52. Bukhari, A. I., and Zipser, D. (1973) *Nat. New Biol.* **243,** 238–241
53. Grodberg, J., and Dunn, J. J. (1988) *J. Bacteriol.* **170,** 1245–1253
54. Gottesman, S. (1989) *Annu. Rev. Genet.* **23,** 163–198
55. Vogel, M., Hess, J., Then, I., Juarez, A., and Goebel, W. (1988) *Mol. Gen. Genet.* **212,** 76–84
56. Ludwig, A., Vogel, M., and Goebel, W. (1987) *Mol. Gen. Genet.* **206,** 238–245

Downloaded from http://www.jbc.org/ by guest on October 1, 2020

In Vivo Proteolytic Degradation of the*Escherichia coli* Acyltransferase HlyC

Caterina Guzmán-Verri, Esteban Chaves-Olarte, Fernando Garci?a, Staffan Arvidson and Edgardo Moreno

J. Biol. Chem. 2001, 276:16660-16666.
doi: 10.1074/jbc.M009514200 originally published online February 15, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M009514200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 53 references, 24 of which can be accessed free at
http://www.jbc.org/content/276/20/16660.full.html#ref-list-1

Downloaded from http://www.jbc.org/ by guest on October 1, 2020