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Genetics

TolC, an *Escherichia coli* outer membrane protein required for hemolysin secretion

(*Erwinia chrysanthemi*/protease secretion)

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ABSTRACT **Secretion of *Escherichia coli* α-hemolysin into the medium does not require the cleavage of an N-terminal signal peptide. The specific secretion apparatus was shown to consist of two proteins, HlyB and HlyD, both located in the inner membrane and encoded by genes contiguous to the hemolysin structural gene (*hlyA*). It was proposed that these two proteins constitute a membrane-bound translocator for hemolysin [Mackman, N., Nicaud, J. M., Gray, L. & Holland, I. B. (1986) *Curr. Top. Microbiol. Immunol.* 125, 159–181]. We show here that an *E. coli* outer membrane protein, the TolC protein, encoded by a gene not located in the *hly* cluster, is specifically required for hemolysin secretion. This result suggests that an outer membrane protein might be a component of the secretion apparatus allowing a specific interaction between the inner and the outer membrane.**

Extracellular proteins produced by Gram-negative bacteria have to cross the cell envelope, which is constituted by two membranes separated by a periplasmic compartment containing the peptidoglycan cell wall (1). Some proteins are translocated through the cytoplasmic membrane by a signal-peptide-dependent step and are then transported out of the cell by a process that involves several specific secretion proteins located in the cell envelope.

Recent studies have shown that several cytotoxins such as *Escherichia coli* hemolysin (2), *Pasteurella haemolytica* leukotoxin (3), and *Bordetella pertussis* adenylate cyclase (4) do not have a signal peptide. Among these toxins, the α-hemolysin of *E. coli* is the most extensively studied (5). The production of active extracellular hemolysin requires four gene products. The hemolysin structural gene *hlyA* encodes an inactive 107-kDa hemolysin which is converted in the cytoplasm to an active form by the action of the 18-kDa *hlyC* product, which is not essential for hemolysin secretion. The hemolysin secretion apparatus consists of at least two membrane proteins, HlyB and HlyD. The HlyA protein contains a secretion signal located at the C terminus, probably within the last 27 amino acid residues (2).

The *P. haemolytica* leukotoxin is homologous to hemolysin and does not have an N-terminal cleavable signal sequence. The genes *lktB* and *lktD*, essential for leukotoxin secretion, show a high degree of identity, 90% and 75%, with *hlyB* and *hlyD*, respectively (3).

The *B. pertussis* cyclolysin is a bifunctional protein with both adenylate cyclase and hemolytic activities. Two secretion genes, *cyaB* and *cyaD*, are respectively homologous to *hlyB* and *hlyD* of *E. coli*, and a third gene, *cyaE*, is also necessary for cyclolysin secretion (4).

The *Serratia marcescens* extracellular metalloprotease (6) and the *Erwinia chrysanthemi* extracellular proteases B and C are also synthesized without N-terminal signal peptides (7), but in contrast to leukotoxin and cyclolysin, these proteins do not have sequence homology with *E. coli* hemolysin, except for a highly conserved region of glycine-rich repeats present in all three of the toxins described above (8) and in these proteases (6, 7). Preliminary results suggest that information sufficient for *Er. chrysanthemi* protease B secretion is located in the last C-terminal 50 amino acid residues of the protein. The cognate secretion genes are clustered on a 5.5-kilobase (kb) DNA fragment (9) and constitute an operon (10) which encodes three secretion proteins, PrtD, PrtE, and PrtF (11), and a protease inhibitor (12). Two of the genes required for protease secretion, *prtD* and *prtE*, have a significant degree of sequence identity (25%) with the *hlyB* and *hlyD* genes. We found it surprising that two genes seem to be sufficient for secretion of hemolysin and leukotoxin, whereas three such genes are required for secretion of *B. pertussis* cyclolysin and *Er. chrysanthemi* proteases. Furthermore, the third protease secretion gene, *prtF*, has 20% identity with the *cyaE* gene of *B. pertussis* (11). This led us to wonder whether secretion of *E. coli* hemolysin might not also require a third secretion function. Our search for additional hemolysin secretion genes was guided by the observation that the third gene which is essential for protease secretion exhibited a significant degree of sequence identity to the *E. coli tolC* gene (11). We demonstrate in this work that the minor outer membrane protein TolC (13) is absolutely required for hemolysin secretion.

MATERIAL AND METHODS

Bacterial Strains, Plasmids, and Bacteriophages. All bacterial strains were derived from *E. coli* K-12. The wild-type strain was C600, which is F⁻ *thr leu fhuA lacY rpsL supE* (from our laboratory collection). The parental *tolC*::Tn*5* strain was GC7442, from the collection of T. Ogura (Kumamoto University Medical School, Kumamoto, Japan). The parental *tolC*::Tn*10* strain GC7459 is described in ref. 14. The strain GC2070 is a spontaneous *tolC* mutant resistant to colicin E1 of strain AB1157 (ref. 15, p. 504). All three *tolC* strains and AB1157 were kindly provided by R. d'Ari and A. Jaffé (Institut Jacques Monod, Paris). Strain SC44 was constructed by phage P1 transduction of the *tolC*::Tn*5* insertion from GC7442 into the C600 recipient strain. Strains POI1734TR and MM8820 are described in ref. 16. The *ompC*::Tn*5* strain pop3496 and the *ompF*::Tn*5* strain pop3495 are both derivatives of strain MC4100, which is F⁻ *araD139* Δ*lac*U169 *rpsL relA thi*. The *ompB101* strain pop1389 and the *envZ11* strain pop1387 are derivatives of pop1010, which is Hfr *his metA rpoB*. These strains are from our laboratory collection. Phage P1 *vir* was used for general transduction (ref. 17, pp. 201–205). Plasmid pANN202-312/2 was described in ref. 18. Plasmid pAX629 was kindly provided by T. Ogura and is described in ref. 19. Plasmid pACYCB2B is a derivative of pACYC184 (20) that carries an Ω insertion (21) in the *tet* gene and codes for spectinomycin resistance (our

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laboratory collection). Plasmids pRUW1 and pRUW4 were described in ref. 9 and plasmid pCHAP231 was kindly provided by A. P. Pugsley (22).

Media. LB and minimal media were described previously (ref. 17, pp. 431 and 433). Red blood cell agar (23), skim milk agar (24), and pullulan indicator agar (25) were also used. To test detergent sensitivity, SDS (1%) was added to LB medium. To select antibiotic-resistant transductants or transformants, antibiotics were added to media at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 50 μg/ml; kanamycin, 50 μg/ml; spectinomycin, 25 μg/ml; and tetracycline, 10 μg/ml. 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) concentration in the red blood cell agar was 40 μg/ml.

Extraction and Manipulation of Plasmids. Isolation of plasmid and transformation of bacteria were performed as described by Maniatis *et al.* (ref. 15, pp. 88–90 and 250–251).

Operon Fusion Construction. *hlyA–lacZ* operon fusions were made by miniMu dl1734 mutagenesis of pANN202-312/2 in strain POI1734TR as described by Castilho *et al.* in ref. 16. Independent mutants were tested for their LacZ phenotype on red blood cell agar + ampicillin + kanamycin + 5-bromo-4-chloro-3-indolyl β-D-galactoside. Nonhemolytic dark blue (Lac^+) colonies were retained for further analysis. Plasmids carrying the fusions were prepared and used to transform C600. The location of the fusion site in pANN202-312/2 was determined by digestion with restriction endonucleases. The *tolC*::Tn*10* mutation was introduced in C600 harboring wild-type pANN202-312/2 or mutated pANN202-312/2 carrying the *hlyA–lacZ* fusions by P1 transduction of the *tolC*::Tn*10* insertion from GC7459 and selection for tetracycline resistance.

Localization of Enzymes in the Different Compartments. Samples of the cultures grown in LB medium at 37°C were taken during the exponential growth phase when cells reached $OD_{600} = 1$, and they were centrifuged for 15 min at 5000 × *g* in the cold. For hemolysin localization, cultures were grown in LB medium supplemented with $CaCl_2$ (5 mM) (26). The supernatant medium, which contains the extracellular proteins, was assayed for activities of β-lactamase (a periplasmic protein) β-galactosidase (a cytoplasmic protein), and hemolysin (an extracellular protein).

Proteins in culture supernatants were precipitated with 10% trichloroacetic acid as described previously (9).

To release periplasmic proteins, cells were washed once with 100 mM Tris·HCl, pH 8, and subjected to cold osmotic shock (27) followed by centrifugation for 15 min at 10,000 × *g*. The supernatant contained the periplasmic fraction. The pellet of the shocked cells was disrupted by sonication for 10 sec at 50 W in a Branson B12 sonifier. Intact cells were pelleted by centrifugation for 5 min at 5000 × *g*. The supernatant was centrifuged for 1 hr at 15,000 × *g*. The supernatant contained the cytoplasmic fraction while the pellet comprised mostly the total membrane fraction (inner and outer membranes). The pellet was solubilized by incubation at 30°C for 30 min in 100 mM Tris·HCl, pH 8/1% Triton X-100/5 mM ethylenediaminetetraacetate. β-Lactamase activity, β-galactosidase activity, and hemolytic activity were assayed in the different fractions. β-Galactosidase and β-lactamase activities were measured as described in refs. 17 (pp. 352–355) and 28, respectively. Hemolytic activity was measured as described in ref. 26. Results were expressed as percent of the total enzymatic activity found in the wild-type strain. Total cell extracts were prepared by heating whole cells to 100°C for 5 min in SDS sample buffer.

Electrophoresis. Proteins were analyzed by SDS/PAGE following the technique of Laemmli (29) as modified by Anderson *et al.* (30).

Immunological Techniques. The antibodies to proteases B and C were described previously (9) and the anti-α-hemolysin serum was a gift of C. Geoffroy-Fauvet (Institut Pasteur).

Immunoblotting was performed as described by Burnette (31). The second antibody was a mouse anti-rabbit immunoglobulin G coupled to alkaline phosphatase, and blots were developed by the nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate technique (Promega Biotec).

RESULTS

***tolC* Mutations Lead to a Defect in Hemolysin Secretion.** *E. coli* K-12 strain C600 was transformed with pANN202-312/2, which carries the hemolytic determinant (18). When grown on blood agar plates colonies of this strain are surrounded by zones of hemolysis. A *tolC*::Tn*5* insertion was introduced into this strain by P1 transduction and selection of kanamycin-resistant colonies. The resulting strain SC44[pANN202-312/2] was hypersensitive to detergents such as SDS as described for other *tolC* mutants (13). Unlike the parent strain, it failed to show hemolysis zones around the colonies when grown on blood agar plates. To confirm the failure of the *tolC* mutant to secrete hemolysin, the extracellular hemolytic activity was measured in culture supernatants of C600[pANN202-312/2] and SC44[pANN202-312/2] as described in *Material and Methods*. As shown in Table 1, extracellular hemolytic activity could be detected only in the parent strain.

Proteins present in the supernatant were precipitated and analyzed by SDS/PAGE followed by Coomassie blue staining. Fig. 1 lane b shows the presence of a 107-kDa polypeptide in the culture medium of the wild-type strain C600-[pANN202-312/2]. Immunodetection analysis shown in Fig. 2 lane a indicates that the 107-kDa protein is recognized by the anti-hemolysin antibodies. Hemolysin could not be detected in the supernatant of SC44[pANN202-312/2] (Fig. 1 lane a and Fig. 2 lane b). To test whether hemolysin accumulates inside the *tolC* mutant, crude extracts of C600-[pANN202-312/2] and SC44[pANN202-312/2] were prepared as described in *Material and Methods* and were subjected to SDS/PAGE analysis followed by immunodetection with anti-hemolysin antibodies. Fig. 2 shows that hemolysin is detectable inside the cells, indicating that hemolysin is synthesized in the *tolC* mutant. However, the intracellular amount was low. Table 1 shows that intracellular activity corresponds to 10% of the extracellular hemolysin found in the wild-type strain.

The loss of hemolysin secretion in the *tolC* mutant could be due to a polar effect of the Tn*5* insertion on the expression of an unknown gene located downstream from *tolC*. The loss of

Table 1. Distribution of hemolytic activity and marker enzymes in $tolC^+$ and $tolC^-$ strains harboring pANN202-312/2

Fraction	Relevant genotype	Activity, % of wild-type total		
		Hemolysin	β-Lactamase	β-Galactosidase
Supernatant	$tolC^+$	90	2	1
	$tolC^-$	<1	2	1
Periplasm	$tolC^+$	5	88	15
	$tolC^-$	5	91	10
Total membrane	$tolC^+$	1	5	2
	$tolC^-$	<1	2	1
Cytoplasm	$tolC^+$	5	5	82
	$tolC^-$	5	5	88

C600[pANN202-312/2] and SC44[pANN202-312/2] strains were cultivated and fractionated, and the different enzymatic activities were measured in the supernatant, periplasm, total membrane fraction, and cytoplasm. The different activities are expressed as percent of the total activity of each enzyme found in the wild-type C600-[pANN202-312/2].

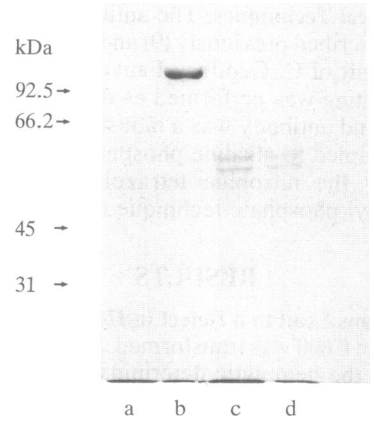

FIG. 1. Extracellular enzymes produced by C600 and SC44 harboring different plasmids. Concentrated culture supernatants were subjected to SDS/PAGE analysis followed by Coomassie blue staining. Polyacrylamide concentration was 9%. Lanes: a, SC44-[pANN202-312/2]; b, C600[pANN202-312/2]; c, SC44[pRUW1]; and d, C600[pRUW1]. The amounts loaded on each lane correspond to 700 μl of culture supernatants before the concentration. Supernatants from exponentially growing cultures (OD_{600} = 1) were used.

hemolysin secretion was also observed in a *tolC* spontaneous mutant (GC2070) after introduction of pANN202-312/2 (Fig. 3). The isogenic but *tolC*$^+$ strain AB1157 secreted hemolysin when harboring this plasmid. To confirm the role of the TolC protein itself, plasmid pAX629, which carries a 1.9-kb chromosomal DNA fragment encoding the TolC protein only (19), was introduced into the SC44[pANN202-312/2] strain and in GC2070[pANN202-312/2] by transformation and antibiotic selection. The transformants were tested for detergent resistance and hemolytic activity. In all the transformants both phenotypes were restored, and α-hemolysin was detected in the culture supernatant as shown in Fig. 3. The amount of hemolysin detected in the supernatant of GC2070[pANN202-312/2, pAX629] was similar to that found in the *tolC*$^+$ strains C600 or AB1157 harboring pANN202-312/2 (Fig. 3). The amount found in the supernatant of SC44[pANN202-312/2, pAX629] was lower (Fig. 3), corresponding to 20% of the extracellular hemolysin found in the wild-type strain as estimated by hemolytic activity measured in the supernatant (data not shown). To test whether the two *tolC* mutations

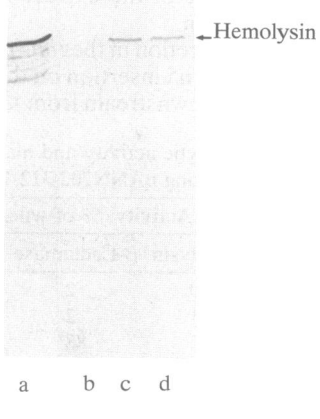

FIG. 2. Immunodetection of hemolysin in culture supernatants and cell extracts of different strains. Concentrated culture supernatants and cell extracts were prepared, subjected to SDS/PAGE (9% acrylamide) analysis, and subsequently transferred onto nitrocellulose. Antibodies against hemolysin were used at a dilution of 1/200. Lanes: a, supernatant of C600[pANN202-312/2]; b, supernatant of SC44[pANN202-312/2]; c, cell extract of C600[pANN202-312/2]; d, cell extract of SC44[pANN202-312/2]. The amounts loaded on each lane correspond to 700 μl of the cultures prior to concentration.

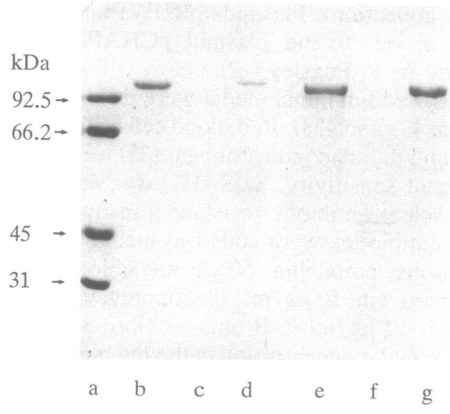

FIG. 3. Extracellular hemolysin produced by TolC$^+$ and TolC$^-$ strains harboring several plasmids. Culture supernatants (700 μl) were used as described in the legend to Fig. 1. Lanes: a, molecular mass markers; b, C600[pANN202-312/2]; c, SC44[pANN202-312/2]; d, SC44[pANN202-312/2, pAX629]; e, AB1157[pANN202-312/2]; f, GC2070[pANN202-312/2]; and g, GC2070[pANN202-312/2, pAX629].

were complemented in trans by pAX629 or in cis by recombination and marker exchange between pAX629 and the chromosome, we cured SC44[pANN202-312/2, pAX629] and GC2070[pANN202-312/2, pAX629] of pAX629. Plasmid pACYCB2B was introduced into these two strains by transformation and antibiotic selection. pACYCB2B and pAX629 belong to the same incompatibility group and are not maintained in the same cell in the absence of antibiotic selection. Spectinomycin- and ampicillin-resistant colonies were purified twice on LB plates containing spectinomycin and ampicillin but not chloramphenicol. Under these conditions, all the colonies tested showed a TolC$^-$ phenotype (detergent sensitivity and no hemolytic activity). This result indicates that the TolC protein itself [the only protein encoded by pAX629 (19)] is necessary for hemolysin secretion. On the other hand, the lower complementation of the *tolC*::Tn*5* insertion than the *tolC* point mutation by pAX629 might indicate a polar effect of the insertion on a downstream gene involved in *tolC* function or expression.

TolC Protein Is Specifically Required for Hemolysin Secretion. The TolC protein is an outer membrane protein and *tolC* mutations are highly pleiotropic, affecting the expression of many unlinked and unrelated genes. The simplest hypothesis was that the outer membrane in the *tolC* mutant was highly disturbed, broadly preventing protein secretion. This was examined by testing the secretion of the *Er. chrysanthemi* proteases B and C. C600 and SC44 strains harboring pRUW1, which carries protease B and C determinants, were grown as described in *Material and Methods*. Culture supernatants were precipitated and analyzed by SDS/PAGE followed by Coomassie blue staining (Fig. 1) or immunoblot analysis with antibodies directed against proteases B and C (Fig. 4). Figs. 1 and 4 show that there is no difference between *tolC*$^+$ and *tolC*::Tn*5* strains with respect to protease secretion. This could be the result of a complementation of the *E. coli* outer membrane defect by the *Er. chrysanthemi* protease secretion function analogous to the TolC protein. However, SC44 strain carrying pRUW1 was still SDS sensitive, and the introduction of the *Er. chrysanthemi* protease secretion genes on a compatible plasmid pRUW4 in strain SC44-[pANN202-312/2] does not restore the hemolytic phenotype. The secretion of *Klebsiella pneumoniae* pullulanase was also unaffected by the presence of the *tolC* mutation (data not shown).

The Major Outer Membrane Proteins Regulated by *tolC* Gene Product Are Not Involved in the Nonhemolytic *tolC*

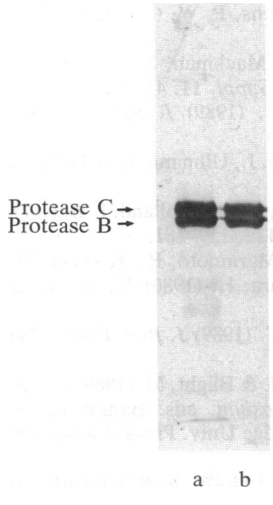

FIG. 4. Immunodetection of proteases in culture supernatants of different strains. Samples (700 μl) of the culture supernatants prepared as described in the legend to Fig. 1 and *Material and Methods* were subjected to SDS/PAGE (9% polyacrylamide) analysis and subsequently transferred onto nitrocellulose. Antibodies against proteases were used at a dilution of 1:5000. Lanes: a, SC44[pRUW1]; b, C600[pRUW1].

Mutant Phenotype. *tolC* mutations are known to prevent the expression of one major outer membrane protein, the OmpF protein, and to lead to an overproduction of the OmpC protein (13). pANN202-312/2 was therefore introduced into strains carrying an *ompF*::Tn*5* mutation, an *ompC*::Tn*5* mutation, the *ompB101* mutation in the *ompR* gene (which prevents the expression of both *ompF* and *ompC*) (32), or the *envZ11* mutation (which prevents *ompF* expression and leads to an overexpression of *ompC*) (33). None of these mutations prevents hemolysin secretion, as shown in Fig. 5. Hence the requirement for *tolC* in hemolysin secretion is not an indirect consequence of its effect on major porin expression.

Hemolysin Location in *tolC* Mutants. To determine where the hemolysin accumulates in the *tolC* mutant, the different cellular compartments of C600[pANN202-312/2] and SC44-[pANN202-312/2] were prepared as described in *Material and Methods*. No difference was observed between the wild type and the mutant in the marker distribution between cytoplasm, supernatant, and periplasm (Table 1). Table 1 shows that C600[pANN202-312/2] secreted 90% of total hemolytic activity into the medium and the remainder was in the periplasm and cytoplasm. In SC44[pANN202-312/2] all the activity was intracellular, being detected both in the cytoplasm and in the periplasm. However, this total intracellular activity was not higher than the total intracellular activity of the wild-type strain and represented only 10% of the extracellular activity found in the wild-type producing strains.

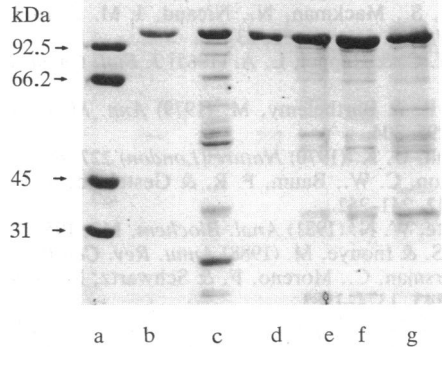

FIG. 5. Extracellular hemolysin produced by several *E. coli* mutants harboring pANN202-312/2. Samples (700 μl) of culture supernatants were used as described in the legend to Fig. 1. Lane a, molecular mass markers. The strains harboring pANN202-312/2 were in the following lanes: b, pop1010; c, MC4100; d, pop1371 (*OmpB101*); e, pop1378 (*EnvZ11*); f, pop3495 (*ompF*::Tn*5*); g, pop3496 (*ompC*::Tn*5*).

The Effect of the *tolC* Mutation on the *hly* Determinant Is Not Transcriptional. Since an intracellular accumulation of hemolysin was not observed in *tolC* mutants, it was not possible to state that the *tolC* mutation affects the secretion step rather than the expression of the *hly* determinant, particularly because *tolC* mutations are known to affect the expression of membrane proteins such as OmpF and OmpC (14). To determine at which level the *tolC* mutation affected the *hly* determinant *lac* operon fusions within pANN202-312/2 were generated in *E. coli* with a miniMu bacteriophage transposon that carries a promoterless *lacZ* gene at its 5′ end (see *Material and Methods*). Insertions leading to a nonhemolytic Lac$^+$ phenotype were further analyzed. The position and orientation of the miniMu *lac* insertions within pANN202-312/2 were determined by *Bgl* II and *Sal* I restriction analysis of plasmid DNA (data not shown). We used two independent fusions in which the miniMu *lacZ* was inserted within the *hlyA* gene and *lacZ* was under the *hlyC* promoter: *hlyA–lacZ* 41 and *hlyA–lacZ* 81. The *tolC*::Tn*10* insertion was introduced in C600 carrying either pANN202-312/2 or the mutated pANN202-312/2 by P1 transduction and selection of tetracycline-resistant colonies. The resulting strain C600 *tolC*::Tn*10*[pANN202-312/2] was SDS sensitive and nonhemolytic, showing that the *tolC*::Tn*10* insertion had the same phenotype as the *tolC*::Tn*5* insertion. The C600 *tolC*::Tn*10* strains harboring pANN202-312/2 that carries *hlyA–lacZ* fusion 41 or 81 were still Lac$^+$. Table 2 shows that the production of β-galactosidase was unaffected by the presence of the *tolC* mutation.

DISCUSSION

In Gram-negative bacteria, protein secretion into the medium involves functions located in the cell envelope and specific for each secreted protein or group of proteins (1). For many extracellular proteins, the existence of such additional functions was suspected when their genetic determinants were cloned from the original protein-secreting bacteria into *E. coli*, where the corresponding proteins were produced but not secreted in the medium. The cloning of the genes that enable these bacteria to secrete the protein led to the discovery of the different proteins forming the specific secretion apparatus. Often, the genes that encode the specific secretion proteins are linked to the structural gene for the extracellular protein, as is the case for the α-hemolysin of *E. coli*, whose secretion is dependent upon two specific membrane proteins, HlyB and HlyD, encoded by genes located close to the structural gene *hlyA* (5). The participation of additional factors encoded by genes that are not contiguous to the *hly* cluster would not have been detected by the procedure described above (cloning the genetic determinants in *E. coli*), probably because such genes would be present in all *E. coli* strains (hemolytic or not). Nonhemolytic mutants of *E. coli* carrying chromosomal mutations unlinked to the *hly* locus were reported but not further studied (23).

Table 2. Effect of the *tolC*::Tn*10* mutation on the *hlyA–lacZ* fusions

Strain	β-Galactosidase activity
C600[pANN202-312/2]	10
C600 *tolC*::Tn*10*[pANN202-312/2]	12
C600[pANN202-312/2 *hlyA–lacZ* 41]	1200
C600[pANN202-312/2 *hlyA–lacZ* 81]	900
C600 *tolC*::Tn*10*[pANN202-312/2 *hlyA–lacZ* 41]	1110
C600 *tolC*::Tn*10*[pANN202-312/2 *hlyA–lacZ* 81]	950

The strains were grown in ML medium. β-Galactosidase activity was assayed by the method of Miller when the culture reached OD_{600} = 1. Activities are expressed as Miller units (ref. 17, p. 352).

In the present work we report that the TolC protein, a minor *E. coli* outer membrane protein (12, 34), is required for hemolysin secretion. This requirement seems to be specific, since TolC is not involved in the secretion of other extracellular proteins tested such as *K. pneumoniae* pullulanase or *Er. chrysanthemi* proteases. Moreover, TolC was not reported to play a role in the general *E. coli* export machinery (35). Furthermore, we have shown that the major outer membrane proteins whose production is regulated by TolC (14) do not play a special role in hemolysin secretion. Finally, using *hlyA–lacZ* operon fusions, we have shown that *tolC* is not involved in *hlyA* transcription. Thus it seems likely that TolC protein has a direct and specific effect on hemolysin secretion.

The attempt to localize intracellular hemolysin in the *tolC* strain did not help to identify a precise function for the TolC protein in the secretion process. Indeed, the total intracellular levels of hemolysin were low, similar to those found in *hlyB* and *hlyD* mutants (36). It is possible that these low intracellular hemolysin levels in all the secretion mutants result from a high instability of internal hemolysin due to proteolytic degradation (36). On the other hand, several experiments indicate that when secretion is blocked by the absence of the export genes the accumulation of intracellular toxin is low, suggesting a coupling between secretion and hemolysin production (8).

It was proposed that hemolysin is directly extruded to the medium without a periplasmic intermediate by the HlyB and HlyD complex at junctions between the inner and the outer membrane or through specific inner membrane vesicles coated with HlyB and HlyD proteins that fuse with the outer membrane (2). However, both proteins are found mostly in the inner membrane (2). In this respect the role of the TolC protein, which is an outer membrane protein, might be to allow the interaction of the HlyB and HlyD complex with the outer membrane in a specific manner. Further work including biochemical and genetical approaches is needed to demonstrate a direct interaction between TolC, HlyB, and HlyD proteins.

Er. chrysanthemi proteases B and C and *B. pertussis* cyclolysin are also secreted by signal-peptide-independent pathways (4, 7). Their secretion machineries are composed of two proteins homologous to HlyB and HlyD proteins, the PrtD and PrtE proteins, respectively, and a third one, the PrtF protein, showing sequence homology with TolC protein in the case of *Er. chrysanthemi* proteases (11). The third secretion component of cyclolysin, the CyaE protein, does not have distinguishable sequence homology with the TolC protein but shows sequence homology with the prtF protein. Yet the CyaE protein, which is most likely an envelope protein (4), might function as a TolC protein analogue. Alternatively, another protein homologous to TolC protein might exist in *B. pertussis* and might be necessary for cyclolysin secretion. Localization of these secretion proteins in the cell envelope will help us to compare their functions. Studies of cyclolysin secretion in *E. coli* might also help us to understand CyaE function.

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