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Genome-Wide Screens: Novel Mechanisms in Colicin Import and Cytotoxicity

Onkar Sharma[*], **Kirill A. Datsenko**, **Sara C. Ess**, **Mariya V. Zhalnina**, **Barry L. Wanner**, and **William A. Cramer**[†]
Department of Biological Sciences, Lilly Hall of Life Sciences, Purdue University, 915 W. State St., West Lafayette, IN 47907

Abstract

Only two new genes (*fkpA* and *lepB*) have been identified to be required for colicin cytotoxicity in the last twenty-five years. Genome-wide screening using the "Keio collection" to test sensitivity to colicins A, B, D, E1, E2, E3, E7 and N from groups A and B, allowed identification of novel genes affecting cytotoxicity and provided new information on mechanisms of action. The requirement of lipopolysaccharide for colN cytotoxicity resides specifically in the LPS inner-core and first glucose. ColA cytotoxicity is dependent on *gmhB* and *rffT* genes, which function in the biosynthesis of LPS and ECA. Of the *tol* genes that function in the cytoplasmic membrane translocon, colE1 requires *tolA* and *tolR* but not *tolQ* for activity. Pal, which interacts with the Tol network, is not required for cytotoxicity of group A colicins. Except for TolQRA, no cytoplasmic membrane protein is essential for cytotoxicity of group A colicins, implying that TolQRA provides the sole pathway for their insertion into/through the cytoplasmic membrane. The periplasmic protease that cleaves between the receptor and catalytic domains of colE7 was not identified, implying either that the responsible gene is essential for cell viability, or that more than one gene-product has the necessary proteolysis function.

Keywords

ASKA; BtuB; Keio; OmpF; translocon

Protein import and export across cellular membranes involves an assembly of integral membrane polypeptides that provide a conduit for protein transfer across the hydrophobic membrane. Colicin import into bacteria is a model for the study of protein import across bacterial membranes. Colicins are produced by *E. coli* in response to stresses such as nutrient depletion and overcrowding, and kill closely related sibling cells that contain the set of receptors and trans-envelope import proteins, but do not contain the respective immunity protein. Based on known components of the translocation network utilized for cell entry, colicins have been divided into two groups. Group A utilizes the *Tol* network consisting of genes *tolA, tolB, tolQ, tolR* and *pal* (Nagel de Zwaig and Luria, 1967; Davies and Reeves, 1975a; Lazzaroni *et al.*, 2002). Group B employs the *Ton* network comprised of *tonB, exbB* and *exbD* (Davies and Reeves, 1975b; Braun *et al.*, 2002). Loss of the colicin receptors in the outer membrane, proteins in the translocation pathway, or of the immunity protein, are the only known mechanisms of colicin resistance or tolerance.

[†]corresponding author (Phone: 765-494-4956); Fax: 765-496-1189;, waclab@purdue.edu.
[*]Present address: Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115.

The first step in colicin import is binding to its outer membrane primary receptor. The protein component of this primary receptor is known to be BtuB for colicin A, E1, E2, E3 and E7, OmpF for colicin N, and FepA for colicins B and D. Subsequent to binding to the primary binding steps, nuclease E colicins, for which the import process has been studied extensively (Di Masi *et al.*, 1973; Mock and Pugsley, 1982; Benedetti *et al.*, 1989; Bouveret *et al.*, 1997; Garinot-Schneider *et al.*, 1997; Kurisu *et al.*, 2003; Housden *et al.*, 2005; Duche *et al.*, 2006; Loftus *et al.*, 2006; Duche, 2007; Sharma *et al.*, 2007; Yamashita *et al.*, 2008), utilize a second receptor/translocator to accomplish translocation across the outer membrane. This secondary receptor/translocator is OmpF for colicin A, TolC for colicin E1 and OmpF/OmpC for colicins E2, E3 and E7. No secondary receptor/translocator has yet been identified for colicin N and the group B colicins. In addition to the primary receptor and secondary receptor/translocator several other proteins have been identified that are responsible for colicin import across the double membrane of the target cell. A total of ten genes, *btuB*, *iutA*, *ompF*, *ompC*, *tolC*, *tolQRAB* and *tsx* have been identified to play a role in the uptake of group A colicins, while the corresponding number for group B colicins is nine ((*cir*, *exbBD*, *fepA*, *fhuA*, *lepB*, *tonB*, *tsx* (Cascales *et al.*, 2007) and the recently identified *fkpA* (Hullmann *et al.*, 2008)). This number is considerably smaller than the number of proteins showed to be involved in protein transport across the mitochondrial double membrane. In the mitochondrial system, virtually all the pre-proteins traverse the outer membrane through the initial entry gate, the TOM (translocase of the outer membrane) complex that consists of seven polypeptides. The full translocation of the pre-proteins across the inner membrane into the mitochondrial matrix requires 10 more polypeptides (Bohnert *et al.*, 2007; Bolender *et al.*, 2008). Thus, on the one hand, it might be expected that additional components, proteins or lipids as yet unidentified, could be involved in colicin uptake across the *E. coli* double membrane. On the other, it is possible that some gene(s) previously proposed to be required for colicin cytotoxicity are not essential. The “Keio Collection” (www.EcoliHub.org/GenoBase) can be used to clarify this situation.

This collection, a single-gene knockout library of the entire *E. coli* genome (Datsenko and Wanner, 2000; Baba *et al.*, 2006), consists of 3985 strains, in duplicate, inoculated in 96-well plates, each well consisting of a distinct single-gene knockout mutant. In the present study, the collection was screened for the sensitivity of each knockout strain to eight colicins, A, B, D, E1, E2, E3, E7 and N, allowing for the identification of knockout strains that are able to grow in the presence of colicin. These colicin ‘tolerant’ or ‘resistant’ single gene knockout strains were further analyzed by complementation with the respective genes from the “ASKA” orfeome library, which restored the colicin sensitivity to the knockout strain. Since only the genes non-essential for cellular viability are represented in the Keio collection, proteins that are required by colicin but which are also essential for cell growth (e. g. *lepB* for colD) will not be scored in the present genomic screening. It is noted that while a ‘positive hit’ implies that the particular gene is important for the colicin cytotoxicity, a ‘negative hit’, where a gene deletion does not affect colicin activity, is equally significant. A ‘negative hit’ implies that, barring a redundancy in the gene function, the gene is not critical for colicin cytotoxicity. Through this study we have been able to define the requirement of each of the 3985 genes in the Keio collection for colicin cytotoxicity.

RESULTS AND DISCUSSION

Colicin cytotoxicity and receptor function

The minimum colicin concentration at which cytotoxicity is expressed can be measured qualitatively by “spot titers,” the minimum amount of colicin that generates a clear “spot” on a Petri dish covered with sensitive cells. As determined through multiple assays ($n \geq 3$), using colicin whose purity was tested and confirmed by SDS-PAGE, and whose concentration was accurately determined (see Experimental Procedures), this concentration

for colE1 is ~125–250 pM, 50–100 pM for colicins E2, E3 and E7. For colB, colD and colN, ~500 pM-1 nM generates a clear zone of inhibition. The minimum inhibitory concentration for colA is ~ 2.5 – 5 nM. Spot titers were also used to estimate the receptor binding affinity of E colicins for BtuB (Taylor *et al.*, 1998; Kurisu *et al.*, 2003). Colicin E2, E3 and E7 were neutralized by BtuB at a colicin:BtuB ratio of 1:1, implying a high affinity, i. e., $K_d \sim 10^{-9}$ M (Kurisu *et al.*, 2003). The binding affinity of colE1 for BtuB is significantly lower (Imajoh *et al.*, 1982; Taylor *et al.*, 1998), so that BtuB neutralized colE1 when added at a ratio of ~1:40 (colicin:BtuB). Because of the low binding affinity of colN for OmpF ($K_d = 2 \times 10^{-4}$ M (Evans *et al.*, 1996a; Evans *et al.*, 1996b), neutralization of colN by OmpF could not be measured. Similarly, the binding affinity of colA to BtuB could not be estimated using the cytotoxicity neutralization assay, implying a relatively low affinity of colA toward BtuB.

I. Colicin N—Screening of the Keio collection for genes that are required for colN cytotoxicity yielded a number of new genes (Table 1), all of which are involved in the biosynthesis of lipopolysaccharide (Fig. 1A, B). Knockout of genes *galU, gmhA, gmhB, gmhC, gmhD, rfaH, waaC, waaF, waaG* and *waaP* conferred cellular resistance to colN, as can be seen from analysis of their growth in the presence and absence of colN (Fig. 2A, B). Complementation with the corresponding gene from the ASKA collection restored sensitivity to colN for all of the above strains (data not shown).

(i) gmhA, gmhB, gmhC and gmhD: All of these genes encode for enzymes involved in the biosynthesis of the heptose moiety (ADP-$_{L, D}$-Hep, ▲, Fig. 1A, B) of the lipopolysaccharide from sedoheptulose 7-phosphate, and were found to be essential for colicin N cytotoxicity. All strains were positively complemented with the corresponding genes from the ASKA collection (Fig. 2A) (data not shown for Δ*gmhC* and Δ*gmhD*). Deletion of *gmhB* has been shown to cause a partial defect in LPS synthesis, so that two different populations of LPS exist, heptose-minus and heptose-rich forms, suggesting that another function in the *E. coli* cell can at least partially compensate for the role of GmhB in LPS synthesis (Kneidinger *et al.*, 2002). Knockout of the *gmhB* gene, however, creates resistance to colN (Fig. 2A, Fig. 3D), suggesting that the concentration of heptose-rich complete LPS molecules in the Δ*gmhB* cells is not sufficient for colN activity.

(ii) waaC and waaF: The ADP-heptose moiety (ADP-$_{L, D}$-Hep) is sequentially added to the 3-deoxy-D-manno-oct-2-ulosonic (Kdo) unit by the *waaC* and *waaF* gene products. Deletion of these genes renders the cells completely resistant to colN (Fig. 2A, B). Sensitivity to the colicin is restored when the knockout cells are complemented with the respective genes.

(iii) waaG, waaP and, rfaH: The *rfaH* gene product, which positively regulates the expression of genes *waaB*, *waaG*, *waaR*, *waaP* and *waaQ* (Pradel and Schnaitman, 1991), is required for colN activity so that its deletion results in resistance to colN and complementation restores sensitivity (Fig. 2B). Primary screening and further verification for sensitivity to colN showed that while WaaP is essential, Δ*waaG* cells are partially resistant (Fig. 2B). WaaB, WaaO, WaaR and WaaQ are not required, implying that the effect of *rfaH* deletion on colN cytotoxicity is due to its positive regulation of *waaP* and *waaG* expression. WaaP, a LPS kinase involved in the addition of phosphate to the first heptose (Fig. 1B), is necessary for the sequential action of WaaQ and WaaY. However, *waaQ* deletion does not affect colN cytotoxicity (data not shown) implying that only the phosphorylation of the first heptose by WaaP is necessary for colN cytotoxicity.

(iv) waaG and galU: Deletion of *galU*, which is involved in the production of UDP-glucose, a substrate for WaaG, renders the cells partially resistant (Fig. 2A) to colN. This partial resistance of Δ*galU* cells for colN can be explained by the leaky LPS phenotype of such a strain (Schnaitman and Klena, 1993). As mentioned above, Δ*waaG* cells are also partially resistant to colN (Fig. 2B). Sensitivity of both of these strains to colN is restored upon complementation (Fig. 2B). However, unlike other colN resistant strains, Δ*waaG* cells in the presence of the colicin display a long incubation lag of ~3 hrs before the initiation of significant growth. The reason for this lag in growth is presently unclear. GalU and WaaG together add the first glucose to the LPS inner core and are required for colN cytotoxicity, while WaaO and WaaR, which extend the LPS by adding the next two glucose moieties, are not necessary. Therefore, the LPS inner core along with the first added glucose defines the minimum binding site for colN. In similar screening studies of the "Keio collection" with T7 phage, it was concluded that the first glucose of the inner core is also essential for cytotoxicity of the phage (Qimron *et al.*, 2006).

(v) Deep Rough Phenotype: Mutation in the genes *gmhA*, *gmhC*, *gmhD*, *waaC*, *waaF* and *waaP* cause the "deep rough phenotype" which has alterations in the LPS structure and results in a > 90% reduction in the concentrations of the porins OmpF, OmpC, LamB and PhoE (Schnaitman and Klena, 1993). Thus, resistance of strains with the deep rough phenotype to colN could be attributed to the reduced concentration of porins in the outer membrane. However, all of these strains were efficiently killed by the seven other colicins used in this study, each of which requires an outer membrane receptor and translocator to for passage across the outer membrane.

While OmpF is the primary and sole receptor for colicin N, it serves as the secondary receptor/translocator for colA. Therefore, colA is more susceptible to decreased levels of OmpF (see section II (iv), VI (iii)) and is expected to have a much lower affinity for OmpF than colN. As evidenced by the growth curves of Δ*gmhA* (Fig. 3A), Δ*waaC* (Fig. 3B), Δ*waaF* and Δ*waaP* cells (data not shown), these deep rough LPS mutants were efficiently killed by colA, implying that the decreased sensitivity of these cells to colN is a consequence of an altered interaction with LPS.

II. Colicin A—Primary screening of the genes required for colicin A cytotoxicity led to the identification of several new genes (Table 1). Δ*tolC* cells were completely resistant to colA and sensitivity was restored upon complementation with *tolC* (Fig. 3C, Fig. 4). The strains Δ*gmhB*, Δ*rffT*, and Δ*yciB* showed varying degrees of resistance to colA. Complementation with the corresponding genes from the ASKA collection restored the sensitivity of these strains to wild type levels (Fig. 4). The *cpxA* and *hns* genes, involved in regulating the expression of OmpF (Suzuki *et al.*, 1996; Batchelor *et al.*, 2005; Skerker *et al.*, 2005), and SurA, required for OmpF folding and insertion into the outer membrane (Lazar and Kolter, 1996), are essential for colA activity. The primary screening also identified a requirement of *yeiL* gene for colA cytotoxicity. Although the Δ*yeiL* strain could not be complemented, it was resistant to colA, but sensitive to other group A colicins, E1, E2, E3, E7 and N (data not shown).

(i) rffT: RffT, a TDP-Fuc4NAc:Lipid II transferase (Rahman *et al.*, 2001), catalyzes the synthesis of Und-PP-GlcNAc-ManNAcA-Fuc4NAc (Lipid III), the third lipid-linked intermediate involved in enterobacterial common antigen (ECA) synthesis. ECA is present in the outer membrane of all gram-negative enteric bacteria (Makela and Mayer, 1976; Mayer and Schmidt, 1979; Kuhn *et al.*, 1988). The absence of *rffT* causes a very weak partial resistance to the colicin and complementation restores colA cytotoxicity (Fig. 4). The absence of the genes, *rfe* and *rffM*, which are also involved in the synthesis of the first and second lipid-linked intermediate in the ECA synthetic pathway, did not affect colA activity

(data not shown). Although the role of this gene in colA cytotoxicity is unclear, it is possible that colA interacts weakly with the ECA. Alternatively, the effect of *rffT* on colA might be distinct from its role in ECA synthesis, indicating another function of the gene or a secondary effect of the gene deletion. An additional effect of mutations in the *rffT* gene is accumulation of lipid II, which stimulates the transcription of *degP* that encodes a heat-shock inducible periplasmic protease (Danese *et al.*, 1998). Thus, stimulation of *degP* transcription might induce degradation of the colicin in the periplasmic space, leading to some degree of colicin resistance.

(ii) gmhB: The *gmhB* gene product, a heptose 1,7-bisphosphate phosphatase, is involved in the synthesis of D-glycero-D-manno heptose 1-phosphate in the pathway of LPS synthesis (Kneidinger *et al.*, 2002). The knockout of the *gmhB* gene creates resistance to colA. Complementation of the Δ*gmhB* cells with $gmhB^+$ gene restores the cytotoxicity of colA (Fig. 3D, Fig. 4). However, other strains deficient in LPS synthesis (including Δ*gmhA*) that are resistant to colN, were killed efficiently by colA (Fig. 3A, B). In addition, as mentioned above, deletion of *gmhB* has only a partial defect in the synthesis of LPS core (Kneidinger *et al.*, 2002), implying that an unknown function of *gmhB*, distinct from its function in LPS synthesis, is required for colA cytotoxicity.

(iii) *tolC*: Deletion of the *tolC* gene caused resistance to colA (Fig. 3C, Fig. 4). This is an unexpected result, because TolC had not been recognized as a requirement for colA cytotoxicity. The likely explanation is that *tolC* deletion decreases OmpF expression by approximately 2 orders of magnitude, while at the same time increasing OmpC expression (Misra and Reeves, 1987; Benedetti *et al.*, 1991). Complementation of Δ*tolC* cells with the *tolC* gene restored the sensitivity to colA.

(iv) Genes affecting outer membrane levels of OmpF: The knockout of *surA* and *yciB* genes results in resistance to colA (data not shown). The products of these genes have been hypothesized to be involved in membrane integrity, and their absence causes a reduction in outer membrane levels of OmpA, OmpC, OmpF and LamB porins (Lazar and Kolter, 1996; Niba *et al.*, 2007). The CpxA-CpxR envelope stress response system regulates the levels of OmpF and OmpC in the outer membrane, so that *cpxA* deletion causes a ~20-fold reduction in the level of OmpF (Batchelor *et al.*, 2005). Hns is also involved in the regulation of OmpF expression so that mutations in *hns* can decrease OmpF and increase OmpC levels (Suzuki *et al.*, 1996). Resistance to colA, caused by deletion of these genes, is inferred to be a consequence of their effect on OmpF levels and the relative low affinity of colA for OmpF (also see section VI (iii)). However, all of these strains are killed by colN, implying that they produce sufficient OmpF for colN to exert cytotoxicity and reinforcing the point that the affinity of colN for OmpF is greater than that of colA.

Although colA is able to kill the cells with “deep rough phenotype” in which the OmpF level is reduced to approximately 10% (~10^4/cell) of the wild type levels, Δ*cpxA* cells, in which the amount of OmpF in the outer membranes is ~20 times less (~5,000/cell) than the wild type level, are resistant to the colicin. Thus, it is estimated that colA requires at least 5×10^3 - 10^4 molecules of OmpF in the outer membrane for efficient cytotoxicity.

(v) yeiL: The gene, *yeiL*, was found to be required for colA cytotoxicity so that its deletion caused complete resistance to colA, but not to the other group A colicins tested. YeiL, a member of the CRP-FNR family (Gostick *et al.*, 1999), is required for viability under nitrogen starvation (Anjum *et al.*, 2000). However, this strain could not be complemented by the corresponding genes from the ASKA library. This was anticipated as complementation by a high-copy $yeiL^+$ plasmid, followed by IPTG-induced overproduction, was not productive because the amplified protein was insoluble (Anjum *et al.*, 2000). Alternatively,

non-complementation raises the possibility that more than one gene is missing in the knockout strain and the colicin-resistance was due to the absence of some other gene essential for the cytotoxicity of that colicin. For this reason, the *yeiL* knockout strain must be analyzed further before any conclusion can be made about its function in colA cytotoxicity.

III. Colicin E1—Primary screening (Table 1) and further verification of the strains in which different Tol proteins were deleted revealed that TolR and TolA are essential for colE1 cytotoxicity. However, the requirement for TolQ is only partial (Fig. 5), as its absence does not result in a loss, but only diminished (~5–10 fold), sensitivity, to colE1 (data not shown). The high concentration of colE1 used in the primary screening (10 nM) prevented observation of this small effect of *tolQ* deletion on colE1 cytotoxicity. The Δ*tolQ* strain, however, is resistant to colicins A, E2, E3, E7 and N (Fig. 5, data shown for colE3). Complementation of all the three strains Δ*tolQ*, Δ*tolR* and Δ*tolA* with the respective genes from the ASKA library restored the colicin sensitivity of these strains (Fig. 5).

Previous studies of the role of Tol proteins in colE1 cytotoxicity appear to have yielded contradictory results. TolA (TolII in older literature), TolQ and TolR have been indicated to be important (Lazzaroni *et al.*, 2002), although other studies have inferred an absolute requirement for TolA and TolQ, but not for TolR (Benedetti *et al.*, 1991; Lazdunski *et al.*, 1998). It is noted that due to a weak/defective ribosome binding site, expression of TolR depends on the successful translation of TolQ (Vianney *et al.*, 1996), a requirement that can be bypassed by an increase in affinity of the ribosome binding site of *tolR*. Therefore, it is likely that at least some of the absolute requirement for TolQ, described previously, might be attributed to the polar effect of TolQ on TolR translation. Yet other studies have identified a TolQ point mutant (TolQ66/TPS66) that is insensitive to E1 (Sun and Webster, 1987; Vianney *et al.*, 1994). However, complementation of this TolQ mutant restored envelope integrity of the cells but not sensitivity to colE1 implying a secondary mutation is responsible for the colE1 resistance. In the present study, the Δ*tolQ* strain from the Keio collection was complemented by the *tolQ* ASKA clone (Fig. 5), implying that it is a true *tolQ* knockout. It is also noted that at least five Tol mutants (Tol III, IV, XIII, XIV and XV) have been identified that were sensitive to colE1 but resistant to colicins, A, E2, E3 and K (Davies and Reeves, 1975a). Tol III was later renamed TolB, while the Tol IV, XIV and XV mutations were traced to the OmpR locus (Sarma and Reeves, 1977). A similar colicin resistance pattern of each of these genes has been detected in the present study. Thus far it has not been possible to determine the relation between Tol XIII and TolQRAB or OmpR but, given the data in the present study, the simplest inference is that this mutant is a TolQ mutant. It is noted that different colE1 proteins exist that have subtle differences in the sequence when compared to the plasmid colE1 (GeneID:2693967) sequence that was used in the present study. In the pioneering study of Davies and Reeves (1975a) four different E1 protein sources were used. Although the identity/sequence of colE1 used in the previous studies is not always clear, all of these colE1 showed a similar resistance pattern. Finally, it is noted that colicin cytotoxicity determination is dependent on the efficiency of the colicin. ColE1 preparations that have low activity would significantly exaggerate Tol requirement of colicin. ColE1 used in the present studies is cytotoxic at sub-nanomolar concentration in ‘spot titer’ tests.

It has been postulated that, due to the role of RfaH and GalU in LPS synthesis, these proteins affect the export and insertion of TolC into the outer membrane (Wandersman and Letoffe, 1993). Thus, the absence of *rfaH* and *galU* results in a reduced level of TolC in the outer membrane. However, in the present studies all LPS mutants identified to be important for colN, including Δ*rfaH* and Δ*galU*, were sensitive to colE1 (data not shown). The sensitivity of these strains to colE1 implies that the decreased level of TolC in the outer membrane is sufficient for this colicin. Because GalU affects only the export of TolC to the

outer membrane and not the production of TolC, the Δ*galU* strain presumably has normal/sufficient amounts of OmpF in the outer membrane to allow sensitivity to colA.

IV. Colicins E2, E3 and E7; Proteolysis Is Necessary for Import—All strains in the Keio collection except those missing the expected *tolA*, *tolB*, *tolQ*, *tolR*, *btuB* and *ompR* genes were found to be sensitive to these colicins (Table 1). One of the genes that is believed to be required for these colicins, whose identity is not yet known, is that of a protease that would enable the C-domain of these colicins to detach before its entry into the cell. Such a proteolytic event has been identified for colD (de Zamaroczy *et al.*, 2001; de Zamaroczy and Buckingham, 2002), colE2 (Sharma and Cramer, 2007) and colE7 (Shi *et al.*, 2005). Preliminary data suggests that a similar proteolysis occurs in colE3 (see below).

Sequence alignment of the R-/C-domains linker region of colE2 and colE3 with that of colE7 revealed a conserved basic residue Lys450 in colE3 that could functionally replace the proteolytic site Arg447 of colE7 (Shi *et al.*, 2005) and Arg452 of colE2 (Sharma *et al.*, 2007). However, when Lys450 of colE3 was mutated in a triple alanine mutant, E3 K450A/N451A/K452A, there was only a small effect on its cytotoxicity (Fig. 6A). To identify the putative cleavage site in colE3, basic residues in and around the linker region were changed and the relative cytotoxicity of these mutants measured through their effect on the growth of colicin sensitive cells (Fig. 6A, B). Although the triple mutants E3 K450A/N451A/K452A (E3 450–452) and E3 P453A/R454A/K455A (E3 453–455) were as active as the wild type colicin, the hexa-mutant E3 K450A/N451A/K452A/P453A/R454A/K455A (E3 450–455), showed a significant reduction in cytotoxicity (Fig. 6A). To further analyze the hexa-mutant, residues 'Lys-Arg-Asn', containing the proteolytic site of colE2 (Sharma *et al.*, 2007) and colE7 (Shi *et al.*, 2005), were introduced into E3 450–455 in two formats. In the first, 'Lys-Arg-Asn' was introduced within the hexa-mutation at site 451–453 (E3 450–455/451-453E7), and in the other at site 456–458 (E3 450–455/456-458E7), i.e., after the hexa-mutation. Cytotoxicity was restored if 'Lys-Arg-Asn' was introduced within the hexa-mutation, but not following it (Fig. 6B). Thus, the introduction of the colE7 proteolytic site restores cytotoxicity in a site-dependent manner, implying that the reduced activity of the E3 450–455 mutant is due to the loss of a putative proteolytic site contained within the hexa-mutation.

However, the gene product responsible for such a proteolytic event could not be identified. OmpT, an outer membrane protease that can cleave the C-domains of colicin E2 (Duche *et al.*, 2009) and E3 (de M. Zamaroczy, personal communication) was found in the present screening not to be required for the activity of any of the eight colicins tested. Only two possible scenarios can be envisaged under which we would not be able to identify the protease during the screening of the Keio Collection: (a) The protein catalyzing the proteolytic activity is essential for bacterial survival in which case it is not represented in the Keio Collection. A precedent is provided by the *lepB* gene that cleaves colicin D (See section **V** below); (b) More than one protein can catalyze proteolysis of these eight colicins.

V. Colicins B and D—Primary screening identified the genes *fepA*, *tonB*, *exbB* and *exbD* to be important (Table 1) for the activity of colB and colD (data not shown). *lepB* which encodes for the inner membrane protease that has been shown to cleave colD (de Zamaroczy *et al.*, 2001) was not represented in the Keio Collection because it is an essential gene. None of the other 3981 knockout strains tested showed colicin resistance, implying that barring any redundancy of gene function, none of these genes were important for the activity of colicins B and D.

VI. Consequences of New Genes for Import Pathways of Colicins—The present screening of the *E. coli* knockout library has enabled the identification of several new genes

involved in colicin cytotoxicity. However, TolQ, which was inferred to be required for the import of colE1, was found to be non-essential. These findings impact current models of colicin import pathways.

(i) Translocon for Import of Nuclease E Colicins; no requirement for Pal: Colicins or their cytotoxic domains utilize protein networks (translocons) to cross the cell envelope and enter the cell to exert cytotoxicity. A model for the outer membrane translocon has been proposed for the group A nuclease E colicins (Kurisu *et al.*, 2003; Housden *et al.*, 2005; Sharma *et al.*, 2007). BtuB and OmpF are the outer membrane components of this translocon, while the members of the *Tol* network, TolQRAB, form the periplasmic and the inner membrane components. The *Tol* network is involved in maintaining the integrity of the outer membrane so that *Tol* mutants leak periplasmic proteins and are hypersensitive to detergent and drugs (Lazzaroni and Portalier, 1992; Lazzaroni *et al.*, 1999; Llamas *et al.*, 2000; Cascales *et al.*, 2002). One of the defined components of the *Tol* network is the peptidoglycan-associated lipoprotein (Pal) (Lazzaroni *et al.*, 2002; Cascales *et al.*, 2007). Interactions of Pal with TolA and TolB have been established by *in vivo* crosslinking (Bouveret *et al.*, 1995; Cascales *et al.*, 2000). The same region of Pal is involved in binding to TolB and peptidoglycan (Clavel *et al.*, 1998; Ray *et al.*, 2000) so that the interactions of Pal with TolB and peptidoglycan have been indicated to be mutually exclusive (Bouveret *et al.*, 1999). Further, the binding sites of Pal and the colE9 T-domain on TolB were found to overlap to a significant degree, and disruption of the TolB-Pal interaction destabilized the integrity of the outer membrane (Loftus *et al.*, 2006). However, despite these significant interactions of Pal with TolA and TolB and its function in outer membrane stability (Lazzaroni *et al.*, 1999; Cascales *et al.*, 2002), Pal was found in the present studies to be non-essential for the cytotoxic activity of all the group A colicins tested. These results are in agreement with two previous reports that implied Pal is not required for cytotoxicity of colicins A, E1, E2 and E3 (Fognini-Lefebvre *et al.*, 1987; Clavel *et al.*, 1998). However, this result contrasts with the suggestion that Pal is part of the import apparatus for these colicins (James *et al.*, 2002; Bonsor *et al.*, 2008) and a recent report from “spot titer” data, where a partial requirement for Pal was implied for the activity of colicin S4, a Tol network dependent colicin (Arnold *et al.*, 2009).

(ii) BtuB-OmpF interactions; requirement for mobility of BtuB in the outer membrane: BtuB, which has a high binding affinity ($K_d < 10^{-9}$ M) for the nuclease E colicins (Kurisu *et al.*, 2003; Sharma *et al.*, 2007), serves as the primary receptor that captures the colicin from the extracellular medium to concentrate it on the cellular surface where it can diffuse and interact with the secondary receptor/translocator, OmpF. Consistent with their function as the primary and secondary receptor for colicin translocation, the outer membrane of each cell contains ~200–400 copies of BtuB, a density two-three orders of magnitude smaller than that of OmpF, which is present at a level of ~ 10^5 molecules/cell (Nikaido and Vaara, 1987). BtuB with bound colicin E3 has been shown in single molecule studies to have a high level of lateral mobility in the outer membrane (Spector *et al.*, manuscript in preparation). The diffusion constant of OmpF was also determined, and found to be much smaller. The present studies suggest that diffusion of one or more outer membrane receptors has an important role in the function of the colicin translocon. Based on the crystal structures of E2R135 and E3R135 complexed to BtuB (Kurisu *et al.*, 2003; Sharma *et al.*, 2007), in wild-type cells, at any given time there are ~2 OmpF molecules within mobile range of a colicin that is bound to its BtuB receptor. In the case of the “deep rough phenotype” LPS mutants, there is a decrease in the level of OmpF in the outer membrane to 10% of that in the wild type (Schnaitman and Klena, 1993), which translates to less than one OmpF molecule within the range of each colicin bound to BtuB. However, these cells are efficiently killed by colA (Fig. 3), suggesting that BtuB is able to diffuse in the outer membrane sufficiently rapidly to form a functional complex with OmpF.

(iii) Subsequent steps in import through the outer membrane: Passage of the colicin N-terminus through the OmpF pore (Kurisu *et al.*, 2003; Zakharov *et al.*, 2004; Sharma *et al.*, 2007; Yamashita *et al.*, 2008) requires interact with the TolB protein in the periplasm (Bouveret *et al.*, 1997; Garinot-Schneider *et al.*, 1997; Loftus *et al.*, 2006), followed by release of the immunity protein (Duche *et al.*, 2006). Subsequently, the C-domain interacts with the OmpF porin (Zakharov *et al.*, 2006), is proteolyzed by the putative periplasmic protease (Shi *et al.*, 2005; Sharma *et al.*, 2007) and enters the cytoplasm where it exerts its cytotoxic effect.

(iv) Colicin N, OmpF and LPS: Interaction of colN with LPS has been implied in the recent 25 Å structure obtained by electron microscopy of a complex of OmpF and colN, which found colN bound to the outer surface of OmpF (Baboolal *et al.*, 2008). The outer surface of OmpF has been suggested to contain the binding site for LPS (Ferguson *et al.*, 2000; Vandeputte-Rutten *et al.*, 2001; Baboolal *et al.*, 2008), and the binding of colN in this region displaced the LPS. It has been suggested that the import of colN might take place at the OmpF-LPS interface and an interaction between OmpF and the N-terminal helix of the colN pore forming domain has been demonstrated (Baboolal *et al.*, 2008). However, to date there has been no direct evidence of an essential function of LPS in colN cytotoxicity. The present study provides the first conclusive evidence for LPS function in colN cytotoxicity and indicates an interaction of colN with LPS. It is possible that the low affinity of colN for OmpF, indicating differences in the colN translocation mechanism, might merely be the result of insufficient LPS for a colN footprint in the above affinity studies. It is noted that interaction with LPS has been previously found to be important for RTX toxins ApxI and ApxII (Ramjeet *et al.*, 2005), and *galU* has been found to be important for the uptake of these two toxins and affects their interaction with LPS (Ramjeet *et al.*, 2008). The above results also correlate well with the fact that the OmpF specific bacteriophage, K20, requires both OmpF and LPS for its receptor function (Silverman and Benson, 1987). However, K20 but not colN requires WaaB, which functions to add another glucose moiety to LPS inner core after RfaG (Traurig and Misra, 1999), for its activity.

(v) Colicin A, OmpF and TolC: Deletion of *tolC* resulted in complete cellular resistance to colA (Fig. 3C, Fig. 4), in contrast to the small effect on colA activity found in previous studies (Benedetti *et al.*, 1991). This effect of *tolC* deletion on colA cytotoxicity was attributed to *tolC*-mediated control of OmpF (Misra and Reeves, 1987). However, colN was able to kill Δ*tolC* cells (Fig. 3C), implying it has a higher affinity for OmpF than colA, which enables colN to bind OmpF even when the outer membrane has a significantly lower OmpF population. This observation is consistent with the conclusion that colA uses OmpF as a secondary translocator while colN uses OmpF as the primary receptor. Because *tolC* deletion decreases OmpF, but increases OmpC, levels in the outer membrane, Δ*tolC* cells are sensitive to colE2, E3 and E7 that can utilize either OmpF or OmpC as the secondary receptor (Sharma *et al.*, 2007).

CONCLUSIONS

The Keio collection and the ASKA library were used to provide a more complete description of the cellular network that supports colicin cytotoxicity. The major findings of the present study are: (a) ColN is unique in that it requires LPS inner core biosynthesis genes for its activity. The LPS inner core along with the first added glucose forms the minimum binding site for colN during its import into the cell. (b) The gene *gmhB* is required for colA cytotoxicity. Several additional genes affecting colA cytotoxicity exert their effect by decreasing OmpF levels in the outer membrane. (c) ColE1 absolutely requires *tolA* and *tolR*, but not *tolQ*, whose deletion generates only partial resistance. (d) Nuclease E colicins E2, E3, and E7 do not require the peptidoglycan associated lipoprotein (Pal) for their

activity. (e) The 3900+ knockout strains that were killed by the colicins tested in the present study imply that, barring any redundancy in the gene function, these genes are not required for colicin cytotoxicity.

EXPERIMENTAL PROCEDURES

Bacterial Strains

The “Keio collection”, a systematic single-gene knockout library of all non-essential genes in *E. coli*, and the “ASKA” orfeome library, which contains each *E. coli* gene cloned into a plasmid vector, allows rapid screening and detection of genes involved in colicin import and cytotoxicity. The Keio collection was constructed in *E. coli* K-12 BW25113 (*rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568*) (Datsenko and Wanner, 2000; Baba *et al.*, 2006) and the ASKA collection in *E. coli* K-12 AG1 (Stratagene, La Jolla, CA), which is a derivative of DH1: *recA1 endA1 gyrA96 thi-1 hsdR17 glnv44(supE44) relA1* (Kitagawa *et al.*, 2005).

The *E. coli* XL1 Blue strain was used as the host strain for cloning of mutations and deletions. Cloning was done in the pET41b vector such that there was a His8 tag at the C terminus of the protein. *E. coli* BL21(DE3) was the host strain for expressing the protein. In the pET41b vector, protein expression is under the control of a strong isopropyl-1-thio-β-D-galactopyranoside (IPTG)-inducible T7 RNA polymerase promoter. All cultures were grown in LB media or on LB agar plates supplemented with antibiotic when required.

Colicins

Plasmids for colicins B, D and E7 were obtained from K. Postle, M. de Zamaroczy and H. S. Yuan, respectively. Colicin E2 and E7 were cloned between the NdeI/XhoI sites of the expression vector pET41b using standard protocols mentioned previously (Zakharov *et al.*, 2004; Sharma and Cramer, 2007). C-terminal His_8-tagged colicins E2, E3 and E7 were purified by metal affinity chromatography using an Ni-charged iminodiacetic acid-agarose column.

Colicins A (Cavard and Lazdunski, 1979), B (Hilsenbeck *et al.*, 2004), D (de Zamaroczy *et al.*, 2001), E1 (Lindeberg *et al.*, 2000) and N (Cavard and Lazdunski, 1979; Izard *et al.*, 1994) were purified using published protocols. BtuB (Kurisu *et al.*, 2003) and OmpF (Yamashita *et al.*, 2008) were purified as mentioned elsewhere. Colicin E1 was cloned in pT 7-7 vector and *E. coli* BL21(DE3) was the host strain for expressing the E1 protein. Purity of colicins was visually ascertained by SDS-PAGE. The concentration of the proteins was measured using extinction coefficients determined by the Protparam tool (Gasteiger *et al.*, 2005).

Mutagenesis and protein purification

All colicin E3 cloning and mutagenesis was done as previously described (Sharma and Cramer, 2007). Purification of overexpressed colicin E3 constructs was carried out with a Ni-charged iminodiacetic acid-agarose column.

Screening procedure

The *E. coli* knockout mutants were applied as drops on LB agar plates containing colicins and incubated at 30 °C overnight. Colicin sensitive cells were killed by the colicin thus allowing only the resistant cells to grow. As a control to check for cell growth, cells were also applied to plates from which colicin was absent. A total of 3985 non-lethal single-gene knockout strains in the library were screened for colicins A, B, D, E1, E2, E3, E7 and N. Because of the high density of cells applied to the plates, the concentration of colicins

incorporated into the plates was high: 10 nM for colicins B, D and E1; 15 nM for colicins A and N; 20 nM for colicins E2, E3 and E7. The strains showing resistance or tolerance to colicin were then purified and further analyzed for resistance by streaking single colonies of the colicin-resistant strains, identified in the primary screening, on colicin containing LB plates that were incubated at 37 °C. Subsequently, colicin resistant strains were verified by PCR. In some cases, independent verification was also performed by targeted screening of a particular strain from the Keio collection. Because of the high colicin concentration used and redundancy in the gene functions, it is noted that the present search could not have identified all the gene products involved in the cytotoxicity of the colicins tested.

Complementation

The ASKA library is a collection of each of the deleted genes cloned in a plasmid such that their expression is under the control of IPTG. Each ASKA clone has been named as pCA24N::geneX, where geneX stands for the gene that is cloned in the vector pCA24N, e. g. pCA24N::tolA, for the *tolA* ASKA plasmid which was used to complement the Δ*tolA* Keio strain. To verify the results obtained by screening the Keio collection, the strains identified to be colicin-resistant were tested for complementation with plasmids from the ASKA library containing the corresponding gene. As a control, the strains were also transformed with the empty vector. These transformants were then tested for sensitivity to the set of colicins. Successful complementation should restore sensitivity to a colicin. As a positive control, strains with genes known from previous studies to be necessary for colicin activity deleted: Δ*tolQ*, Δ*tolR* and Δ*tolA* for group A colicins A, E1, E2, E3, E7 and N; Δ*btuB* for colicins A, E1, E2, E3 and E7, and; Δ*fepA* for colicins B and D, were complemented with the respective genes and tested for restoration of colicin sensitivity. Complementation was achieved in the presence of 0.5 mM IPTG for all the mutants except the Tol knockouts. In the case of the Tol mutants complementation was achieved in the absence of any IPTG.

Cytotoxicity Assays

The time course for growth of the colicin resistant strains carrying the corresponding ASKA genes (or the vector control) was analyzed in the presence and absence of colicin. 10 μl from an overnight culture was added to 190 μl of LB medium in 96 well plates supplemented with chloramphenicol (25 μg/ml), IPTG (0.5 mM) and colicin A (5 nM) or colicin N (500 pM). The cells were allowed to grow at 37 °C and the growth was monitored by measuring optical density at 410 nm at 30 min intervals.

The cytotoxicity of the different colicin E3 mutant constructs was compared by analyzing the effect of colicin on the growth rate of colicin-sensitive *E. coli* K17 indicator cells. 100 ml of LB media were inoculated with an overnight culture of the indicator cells and grown to an OD_{600} (optical density at 600 nm) of 0.1. The culture was then aliquoted, and a different colicin construct was added to each sample at a concentration of 200 pM. Cell growth was monitored by measuring the optical density at 600 nm at 1 hour intervals.

Cytotoxicity of colicins was assayed qualitatively using a ‘spot titer’, in which a lawn (~2×10^8 cells) of colicin sensitive *E. coli* K17 indicator cells in log growth phase were spread on a LB plate to which different concentrations of colicin are applied as 20 μl drops. After overnight incubation of the plates at 37 °C, killing of the bacterial cells is seen as clear spots in a lawn of bacteria. The lowest inhibitory concentration was defined as the smallest concentration that would generate a clear zone of inhibition.

Colicin Affinity for Receptor; Neutralization by BtuB and OmpF

The receptor binding affinity of colicin was assayed by the ability of BtuB (OmpF for colN) to neutralize the cytotoxicity of the colicin assayed by the microbiological spot titer. BtuB (OmpF for colN) in detergent solution was mixed with the colicin at different molar ratios before addition of a 20 μl aliquot to the Petri plate, on which a lawn of colicin sensitive *E. coli* K17 indicator cells in log growth phase had been spread. The minimum molar ratio of BtuB (OmpF for colN) to colicin that prevented colicin cytotoxic activity was used as an indicator of colicin affinity for the receptor. The concentrations of colicins A, E1, E2, E3, E7 and N, respectively, used for the neutralization experiment were 5 nM, 0.5 nM, 0.5 nM, 0.5 nM, 0.5 nM and 1 nM. At these concentrations, colicins, diluted in detergent containing buffers, were able to generate a clear zone of inhibition in the absence of BtuB.

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Abbreviations

ADP-L	D-Hep, adenosine diphosphate 5′-L-*glycero*-β-D-*manno*-heptose
ASKA library	a complete set of *E. coli* K-12 ORF archive
col	colicin
ECA	enterobacterial common antigen
E2R135	R-domain of colicin E2
E3R135	R-domain of colicin E3 IPTG, isopropyl-β-D-thiogalactopyranoside
LPS	lipopolysaccharide
OD	optical density
PCR	polymerase chain reaction
TOM	translocase of the outer membrane
wt	wild type

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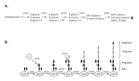

Figure 1.

A. Pathway for synthesis of ADP-L, D-heptose (▲in part B). All genes in this pathway were found to be important for cytotoxicity of colicin N. **B**. Biosynthesis of LPS by addition of successive heptose (▲) and glucose moieties (Δ). The *galU* and *waaG* gene deletion has a partial phenotype, *waaC*, *waaF* and *waaP* genes were required, while *waaO*, *waaQ* and *waaR* were not required for colicin N activity (*waaU* is not represented in the Keio collection). WaaP is involved in phosphorylation of the first heptose (o). Based on the partial phenotype of the *galU* and *waaG* knockouts, it is inferred that colN interacts with the LPS inner core along with the first added glucose.

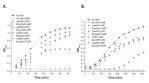

Figure 2.

Effect of colN on the growth of wild type and knock-out strains. **(A)** Knock-out strains Δ*galU*, Δ*gmhA*, Δ*gmhB* and Δ*waaC* strains and their complemented counterpart (**C**Δ*galU*, **C**Δ*gmhA*, **C**Δ*gmhB* and **C**Δ*waaC* **C**Δ*waaF*) were tested for colN sensitivity. **(B)** LPS mutant strains Δ*rfaH*, Δ*waaF*, Δ*waaG* and Δ*waaP* were also resistant to colN and their complemented counterpart (**C**Δ*rfaH*, **C**Δ*waaF*, **C**Δ*waaG* and **C**Δ*waaP*) were tested for colN sensitivity. The growth of these strains in the absence of colicin has not been shown for purposes of clarity. OD_{410} of 0.1, 0.45, 0.7, 0.1 and 1.2 correspond approximately to a cell titer per ml of 0.7×10^8, 2.6×10^8, 5.5×10^8, 25×10^8 and 28×10^8, respectively.

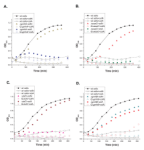

Figure 3.

Comparison of the effect of colA and colN on the growth of wild type and knock-out strains. (**A)** Δ*waaC* cells and **(B)** Δ*gmhA* are resistant to colN, but sensitive to colA. Sensitivity to colN is restored upon complementation of these strains (**C**Δ*gmhA* and **C**Δ*waaC*). Thus, the resistance of these strains to colN is not attributed to the low titers (10% of wt levels) of OmpF in the outer membrane. Rather, it is inferred that LPS plays a direct role in colN cytotoxicity. **(C)** Δ*tolC* cells are resistant to colA, most likely due to the decreased level of OmpF in the outer membrane (2% of wt levels (Misra and Reeves, 1987)) in Δ*tolC* cells. However, due to the higher affinity of colN for OmpF, Δ*tolC* cells are lysed by colN. **D.** Δ*gmhB* cells are resistant to both colN and colA. Sensitivity to these colicins is restored upon complementation (**C**Δ*gmhB*). Because colA is the least potent of all group A colicins and utilizes only OmpF, in contrast to nuclease E colicins which can utilize both OmpF and OmpC, the above strains were not tested individually with nuclease E colicins. The concentration of colA and colN was 5 nM and 0.5 nM, respectively.

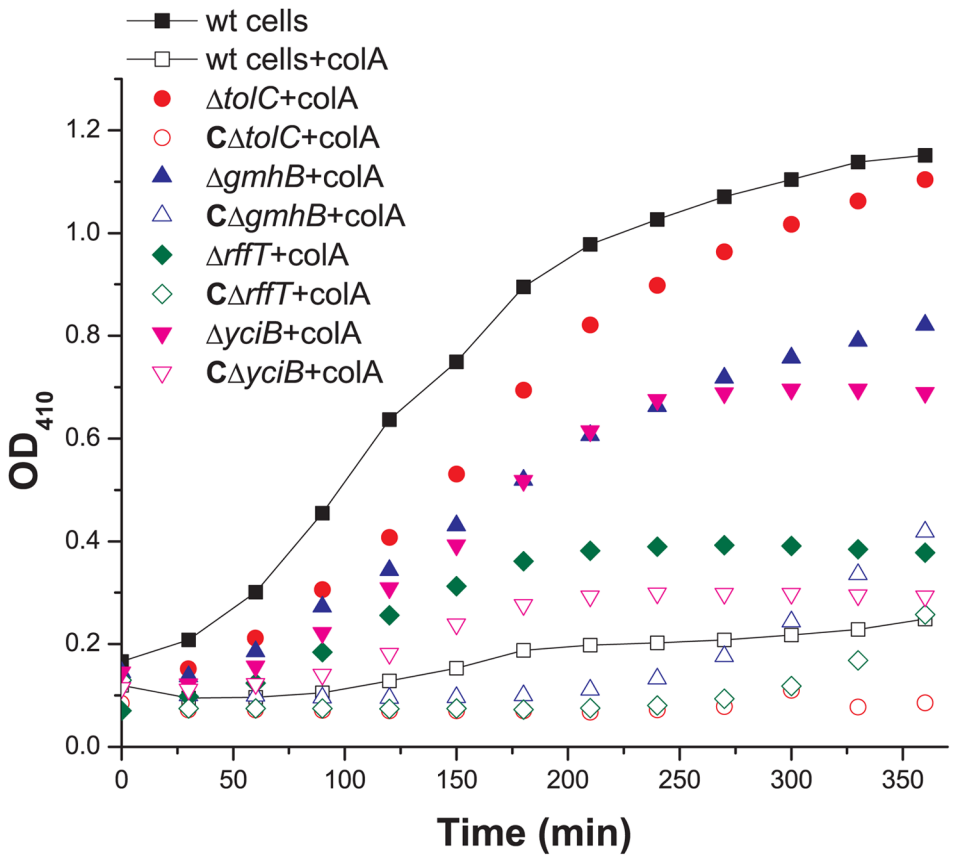

Figure 4.

Effect of colA on the growth of wild type and knock-out strains. Knock-out strains Δ*yciB*, Δ*rffT*, Δ*gmhB* and Δ*tolC* strains and their complemented counterpart (**C**Δ*yciB*, **C**Δ*rffT*, **C**Δ*gmhB* and **C**Δ*tolC*) were tested for colA sensitivity. The knockout strains were resistant to colA. Complementation with the respective genes from the ASKA collection restored their sensitivity. The growth of these strains in the absence of colicin has not been shown for purposes of clarity. It is noted that the **C**Δ*tolC* strain is more sensitive than the wild type cells to colA.

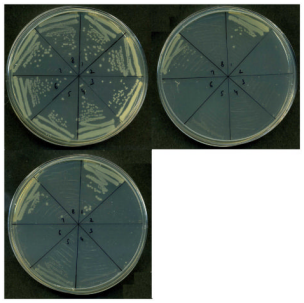

Figure 5.

TolA and TolR are essential for colicin E1 cytotoxicity while TolQ is not. ΔtolA, LB plates containing no colicin (top left), colicin E1 (top right) or colicin E3 (bottom left) were divided into 8 sections each containing different cells. These are: 1) Δ*tolA*; 2) **C**Δ*tolA*; 3) and 4) wt cells containing empty vector; 5) Δ*tolQ*; 6) **C**Δ*tolA*; 7) Δ*tolR*; 8) **C**Δ*tolR*;. Because, IPTG-induced overexpression of TolQ, TolR and TolA interferes with cell growth, IPTG was not added to these plates and complementation occurred due to leaky expression of the ASKA clones.

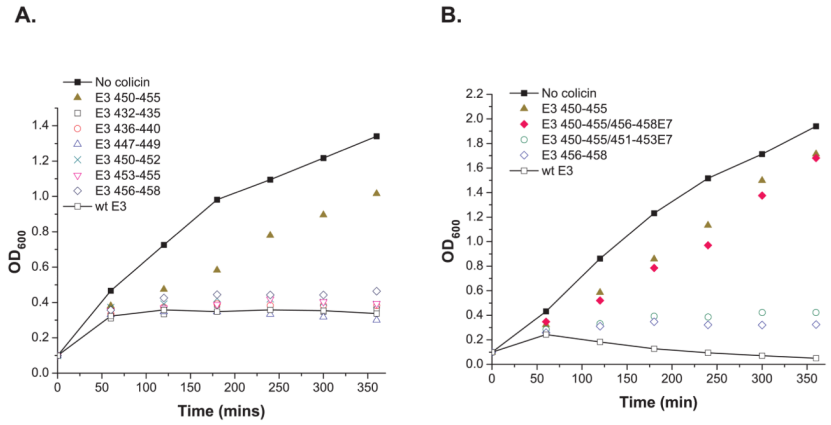

Figure 6.

Cytotoxicity of colicin E3 constructs, with mutations in the R-/C-domain linker region. The cytotoxic activities were compared by analyzing the effect of the E3 constructs on growth of colicin-sensitive cells. **(A)** All of the mutants tested, except the hexa-mutant E3 450–455, had wild type activity. Although mutating the residues 450–452 and 453–455 to alanine did not have any effect on colicin activity, the combination of the two mutations to generate the construct E3 450–455 reduced colicin activity by a factor of ~30, as assayed by spot titer (data not shown). **(B)** When the proteolytic site of colicin E7, residues Lys446-Arg447-Asn448, was mutated into the hexa-mutant, the cytotoxicity was restored in a site dependent manner. Cytotoxicity was restored only if the residues were placed within the hexa-mutation (E3 450–455/451-453E7) and not after it (E3 450–455/456-458E7). Definition of colicin mutants: E3 432–435, colicin E3 with mutations R432A/K433A/K434A/K435A; E3 436–440, colicin E3 with mutations E436A/D437A/K438A/K439A/R440A; E3 447–449, colicin E3 with mutations N447A/D448A/E449A; E3 450–455, colicin E3 with mutations K450A/N451A/K452A/P453A/R454A/K455A; E3 456–458, colicin E3 with mutations G456A/F457A/K458A; E3 450–455/451-453E7, colicin E3 with mutations K450A/N451K/K452R/P453N/R454A/K455A; E3 450–455/456-458E7, colicin E3 with mutations K450A/N451A/K452A/P453A/R454A/K455A/G456K/F457R/K458N.

Table 1

List of genes affecting the cytotoxicity of colicins A, B, D, E1, E2, E3, E7 and N.

Gene*	Mutant	Cellular/Colicin Import Functions	Complementation	Source
		Colicin N		
gmhA	*gmhA740::kan*	LPS core biosynthesis	Yes	This study
gmhB	*gmhB725::kan*	LPS core biosynthesis	Yes	This study
gmhC	*gmhC745::kan*	LPS core biosynthesis	Yes	This study
gmhD	*gmhD 731::kan*	LPS core biosynthesis	Yes	This study
waaC	*waaC 733::kan*	LPS core biosynthesis	Yes	This study
waaF	*waaF 732::kan*	LPS core biosynthesis	Yes	This study
waaG	*waaG 742::kan*	LPS core biosynthesis	Yes	This study
waaP	*waaP741::kan*	LPS core biosynthesis	Yes	This study
rfaH	*rfaH 783::kan*	Regulates the expression of genes *waaB*, *waaG*, *waaR*, *waaP* and *waaQ*	Yes	This study
galU	*galU745::kan*	LPS core biosynthesis	Yes	This study
tolA	*tolA788::kan*	Tolerance to group A colicins; maintains cell envelope integrity	Yes	Previous studies[2] and this study
tolQ	*tolQ786::kan*	Tolerance to group A colicins; maintains cell envelope integrity	Yes	Previous studies[2] and this study
tolR	*tolR787::kan*	Tolerance to group A colicins; maintains cell envelope integrity	Yes	Previous studies[2] and this study
ompF	*ompF746::kan*	Outer membrane porin F	NT	Previous studies[3] and this study
ompR	*ompR739::kan*	Regulates production of OmpF and OmpC	NT	Previous studies[3] and this study
		Colicin A		
gmhB	*gmhB725::kan*	LPS core biosynthesis	Yes	This study
yciB	*yciB762::kan*	Involved in zinc uptake	Yes	This study
rffT	*rffT744::kan*	Involved in enterobacterial common antigen synthesis	Yes	This study
tolC	*tolC732::kan*	Outer membrane component of the tripartite efflux pumps	Yes	Previous studies[4] and this study
hns	*hns746::kan*	Regulates OmpF and OmpC expression	NT	This study
surA	*surA765::kan*	Periplasmic chaperone for OM porins	NT	This study
cpxA	*cpxA771::kan*	Involved in OmpF and OmpC regulation	Yes	This study
yeiL	*yeiL790::kan*	Involved in mid-term, stationary-phase viability under nitrogen starvation.	No[1]	This study
tolA	*tolA788::kan*	Tolerance to group A colicins; maintains cell envelope integrity	Yes	Previous studies[2] and
tolB	*tolB789::kan*	Uptake of some group A colicins	NT	Previous studies[2] and this study
tolQ	*tolQ786::kan*	Tolerance to group A colicins; maintains cell envelope integrity	Yes	Previous studies[2] and this study
tolR	*tolR787::kan*	Tolerance to group A colicins; maintains cell envelope integrity	Yes	Previous studies[2] and this study
ompF	*ompF746::kan*	Outer membrane porin F	NT	Previous studies[3] and this study
ompR	*ompR739::kan*	Regulates production of OmpF and OmpC	NT	Previous studies[3] and this study

Gene*	Mutant	Cellular/Colicin Import Functions	Complementation	Source
btuB	*btuB754::kan*	Receptor for vitamin B_{12} uptake	Yes	Previous studies[5] and this study
		Colicins B and D		
tonB	*tonB760::kan*	Together with OM receptors involved in the uptake of iron and cyanocobalamin	NT	Previous studies[6] and this study
exbB	*exbB779::kan*	Deletion causes tolerance or sensitivity to group B colicins	NT	Previous studies[6] and this study
exbD	*exbD778::kan*	Deletion causes tolerance or sensitivity to group B colicins	NT	Previous studies[6] and this study
fepA	*fepA721::kan*	Ferric enterobactin outer membrane receptor	Yes	Previous studies[6] and this study
		Colicin E1		
tolA	*tolA788::kan*	Tolerance to group A colicins; maintains cell envelope integrity	Yes	Previous studies[2] and this study
tolR	*tolR787::kan*	Tolerance to group A colicins; maintains cell envelope integrity	Yes	Previous studies[2] and this study
tolC	*tolC732::kan*	Outer membrane component of the tripartite efflux pumps	Yes	Previous studies[7] and this study
btub	*btuB754::kan*	Receptor for vitamin B_{12} uptake	Yes	Previous studies[5] and this study
		Colicins E2, E3 and E7		
tolA	*tolA788::kan*	Tolerance to group A colicins; maintains cell envelope integrity	Yes	Previous studies[2] and this study
tolB	*tolB789::kan*	Uptake of some group A colicins	NT	Previous studies[2] and this study
tolQ	*tolQ786::kan*	Tolerance to group A colicins; maintains cell envelope integrity	Yes	Previous studies[2] and this study
tolR	*tolR787::kan*	Tolerance to group A colicins; maintains cell envelope integrity	Yes	Previous studies[2] and this study
ompR	*ompR739::kan*	Regulates production of OmpF and OmpC	NT	Previous studies[3] and this study
btuB	*btuB754::kan*	Receptor for vitamin B_{12} uptake	Yes	Previous studies[5] and this study

*Allele designations, J. Wertz, Coli Genetic Stock Center (CGSC), Yale University NT, Not tested

[1], Overexpression of YeiL leads to insoluble protein (See section II (v))

[2], Group A colicins require the Tol system (Nagel de Zwaig and Luria, 1967; Davies and Reeves, 1975a; Lazdunski *et al.*, 1998; Cascales *et al.*, 2007)

[3], (Mock and Pugsley, 1982; Tommassen *et al.*, 1984; Benedetti *et al.*, 1989; Sharma *et al.*, 2007)

[4], (Benedetti *et al.*, 1991)

[5], (Di Masi *et al.*, 1973; Benedetti *et al.*, 1989; Kurisu *et al.*, 2003; Sharma *et al.*, 2007)

[6], Group B colicins require the Ton system (Davies and Reeves, 1975b; Cascales *et al.*, 2007)

[7], (Nagel de Zwaig and Luria, 1967)