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Functional Interactions between Coexisting Toxin-Antitoxin Systems of the *ccd* Family in *Escherichia coli* O157:H7[▽]

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Toxin-antitoxin (TA) systems are widely represented on mobile genetic elements as well as in bacterial chromosomes. TA systems encode a toxin and an antitoxin neutralizing it. We have characterized a homolog of the *ccd* TA system of the F plasmid (ccd_F) located in the chromosomal backbone of the pathogenic O157:H7 *Escherichia coli* strain (ccd_{O157}). The ccd_F and the ccd_{O157} systems coexist in O157:H7 isolates, as these pathogenic strains contain an F-related virulence plasmid carrying the ccd_F system. We have shown that the chromosomal ccd_{O157} system encodes functional toxin and antitoxin proteins that share properties with their plasmidic homologs: the $CcdB_{O157}$ toxin targets the DNA gyrase, and the $CcdA_{O157}$ antitoxin is degraded by the Lon protease. The ccd_{O157} chromosomal system is expressed in its natural context, although promoter activity analyses revealed that its expression is weaker than that of ccd_F. ccd_{O157} is unable to mediate postsegregational killing when cloned in an unstable plasmid, supporting the idea that chromosomal TA systems play a role(s) other than stabilization in bacterial physiology. Our cross-interaction experiments revealed that the chromosomal toxin is neutralized by the plasmidic antitoxin while the plasmidic toxin is not neutralized by the chromosomal antitoxin, whether expressed ectopically or from its natural context. Moreover, the ccd_F system is able to mediate postsegregational killing in an *E. coli* strain harboring the ccd_{O157} system in its chromosome. This shows that the plasmidic ccd_F system is functional in the presence of its chromosomal counterpart.

Toxin-antitoxin (TA) proteic systems were originally discovered on low-copy-number plasmids (for reviews on TA systems, see references 11, 22, 24, and 29). They are composed of two genes organized in an operon encoding a toxin and an antitoxin that antagonizes it. The expression of the TA genes is autoregulated at the transcriptional level; the antitoxin acts as a repressor and the toxin often as a corepressor. The antitoxin is an unstable protein degraded by an ATP-dependent protease, while the toxin is a stable protein that inhibits an essential cellular process (e.g., replication and translation). TA systems contribute to plasmid stability by a mechanism called postsegregational killing (PSK). PSK relies on the differential stabilities of the antitoxin and toxin proteins and leads to the killing of daughter bacteria that did not receive a plasmid copy at cell division (31, 50, 53).

Recent computational analyses have shown that TA systems are widely represented in eubacterial and archaebacterial chromosomes, suggesting a role for horizontal gene transfer in the spread of these genes (5, 6, 38). The localization of chromosomal TA systems is quite varied. Some are localized within exogenous DNA islands like phages ($relBE_{K\text{-}12}$ in the cryptic lambdoid Qin prophage of *Escherichia coli* MG1655) (40), transposons (*relBE* homolog in Tn*5401* of *Bacillus thuringiensis* (23), and superintegrons (*relBE*, *parDE*, *phd-doc*, and *higAB* homologs in the superintegron of *Vibrio cholerae* [17, 38, 41]). Others, such as *mazEF* (*chpA*) and *chpB* of *E. coli* K-12, are apparently settled in the chromosome, flanked by metabolic or regulatory genes (35, 36). The chromosomal TA systems were shown to be activated under stress conditions, although the outcomes of activation appear to be different for the various systems. On the one hand, the *mazEF* system was shown to be a suicide module leading to cell death under various stressful conditions (e.g., overproduction of ppGpp, DNA damage, high temperatures, and oxidative stress) (2, 4, 30, 46, 47). On the other hand, the *mazEF* and $relBE_{K\text{-}12}$ systems have been described as growth modulators under conditions of amino acid starvation (15, 16, 39). The function of TA systems localized in transposons or superintegrons could be similar to that of plasmidic TA systems, i.e., they could stabilize these exogenous DNA islands within the bacterial genome (17, 41).

We have performed a functional analysis of the chromosomal ccd_{O157} system of *E. coli* O157:H7, which is homologous to the *ccd* TA system of the F plasmid (ccd_F) (for reviews on *ccd*, see references 19 and 52). The ccd_F system is composed of the unstable $CcdA_F$ antitoxin, which is degraded by the Lon ATP-dependent protease (53, 54), and of the stable $CcdB_F$ toxin. Both $CcdA_F$ and $CcdB_F$ are required for autoregulation (1, 42, 49). $CcdB_F$ targets the DNA gyrase and leads to replication and transcription inhibition, SOS induction, and ultimately, to cell death (8, 9, 20, 31, 34). The ccd_F and the ccd_{O157} systems coexist in *E. coli* O157:H7 isolates; the ccd_F system is

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or plasmid properties[a]	Reference or source
Strains		
B462	DH2 *lacI*^{q} *gyrA462 zei*::Tn*10*	Laboratory collection
CSH50	*ara* Δ(*lac-pro*) *strA thi*	37
SG22622	MC4100 *cpsB*::*lacZ* Δ*ara malP*::*lacI*^{q}	S. Gottesman
SG22622 *gyrA462 zei*::Tn*10*	SG22622 containing the *gyrA462* $CcdB_F$ resistance mutation	3
SG22623	SG22622 Δ*lon-510*	S. Gottesman
O55:H7 TB182A	Enterohemorrhagic *E. coli*	STEC center
O55:H7Δccd_{O157}	O55:H7 TB182A Δccd_{O157}	This work
Plasmids		
pBAD33	p15A, Cm^r, pBAD promoter	28
pBAD33-$ccdB_F$	pBAD33 derivative containing the $ccdB_F$ gene under the control of the pBAD promoter	This work
pBAD33-$ccdB_{O157}$	pBAD33 derivative containing the $ccdB_{O157}$ gene under the control of the pBAD promoter	This work
pCP20	pSC101 ts derivative containing the FLP gene under the control of the λ *c*I857 repressor	13
pJL207	p15A, Cm^r, *lacZ*	33
pJL-ccd_F (pULB2600)	pJL207 containing the OP of the ccd_F operon	42
pJL-ccd_{O157}	pJL207 containing the OP of the ccd_{O157} operon	This work
pKD4	pANTSγ derivative containing an FRT-flanked kanamycin resistance gene, Amp^r	21
pKK223-3	ColE1, Amp^r, pTac promoter	10
pKK-$ccdA_F$ (pULB2709)	pKK223-3 derivative containing the $ccdA_F$ gene under the control of the Tac promoter	53
pKK-$ccdA_{O157}$	pKK223-3 derivative containing the $ccdA_{O157}$ gene under the control of the Tac promoter	This work
pKOBEG	pSC101 ts derivative containing an arabinose-inducible λ redγβα operon, Cm^r	12
pKT279	pBR322 derivative, Tet^r	48
pKT-ccd_F (pULB2707)	pKT279 containing the ccd_F operon	42
pKT-ccd_{O157}	pKT279 containing the ccd_{O157} operon	This work
pMLO59	pGB2 ts derivative, Spec^r	M. Labocka
pMLO-ccd_F (pULB2710)	pMLO59 containing the ccd_F operon	53
pMLO-ccd_{O157}	pMLO59 containing the ccd_{O157} operon	This work

[a] FRT, FLP recombination target; ts, temperature sensitive.

present on an F-related virulence plasmid (pO157), while the ccd_{O157} system is present on the chromosome. Therefore, we have tested whether the components of these systems interact and showed that the ccd_F system is functional in the presence of its chromosomal homolog.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Strains and plasmids used in this work are listed in Table 1.

Construction of the O55:H7Δccd_{O157} strain. The pKOBEG plasmid (12) was used as described at the website http://www.pasteur.fr/recherche/unites/Ggb/3SPCRprotocol.html. The kanamycin resistance cassette of pKD4 was amplified by PCR with the following primers: P1 (5′ ATA CTA GAC GTA TAA ATT GTA CAG GAG CAC GAT ATC GTG TAG GCT GGA GCT GCT TC) and P2 (5′ AAG GAT TTG GGT GAG GGA GAG GCG GTC GCG TCT TAA CAT ATG AAT ATC CTC CTT AG). Deletion of the ccd_{O157} system was constructed by following the method described in reference 21. The deletion and the flanking regions were checked by DNA sequencing.

Construction of plasmids. (i) Expression plasmids. The expression plasmids are isogenic, i.e., all the open reading frames (ORFs) were cloned using the same restriction sites in the expression vectors, and all the regulatory sequences were added by PCR (the Shine-Dalgarno box and the sequence between the Shine-Dalgarno box and the ATG of the ORFs were identical).

To construct the pBAD-$ccdB_F$ plasmid, the *ccdB* gene from the F plasmid was amplified by PCR using pULB2250 as a template (8) and the following primers: 5′CcdB-XbaI (5′TCT AGA AGG AGG GTG AAA TGC AGT TTA AGG) and 3′CcdB-PstI (5′AGT CTC TGC AGT TAT ATT CCC CAG AAC). The PCR product was cloned into the TOPO-XL vector (Invitrogen). The resulting plasmid was then digested by XbaI and PstI. The fragment containing $ccdB_F$ was inserted into pBAD33 that was opened by the same enzymes. The ligation mixture was transformed in strain B462.

To construct the pBAD-$ccdB_{O157}$ plasmid, the *ccdB* gene from *E. coli* O157:H7 was amplified by PCR using *E. coli* O157:H7 chromosomal DNA (ATCC 700927) as a template and the following primers: 5′$CcdB_{O157}$-XbaI (5′TCT AGA AGG AGG TAG CGA TGC AAT TTA CGG) and 3′$CcdB_{O157}$-PstI (5′AGT CTC TGC AGT TAA ATC CCG TCG AGC). The PCR product was digested by XbaI and PstI and inserted into pBAD33 that was digested with the same enzymes. The ligation mixture was transformed in strain B462.

To construct the pKK-$ccdA_{O157}$ plasmid, the *ccdA* gene from *E. coli* O157:H7 was amplified by PCR using *E. coli* O157:H7 chromosomal DNA (ATCC 700927) as a template and the following primers: 5′$CcdA_{O157}$-EcoRI (5′TTG TGA ATT CTA TGA CTG CAA AAC GTA CCA) and 3′$CcdA_{O157}$-PstI (5′AGT CTC TGC AGC TAG AAG CTC CGG TAC TC). The PCR product was cloned into the TOPO-XL vector (Invitrogen). The resulting plasmid was then digested by EcoRI and PstI. The fragment containing $ccdA_{O157}$ was inserted into pKK223-3 that was opened by the same enzymes.

(ii) Promoter activity plasmids. To construct the pJL-OPccd_{O157} plasmid, the operator/promoter region of the ccd_{O157} operon was amplified by PCR using the Topo-*folA*ccd_{O157} plasmid (see below) as a template and the following primers: 5′O/P2-ccd_{O157} (5′GGT ATT CAG CGA ATT CCA CGA CGC TG) and 3′O/P2-ccd_{O157} (5′TCA GCA TTG AGC GCA ACC GTA AGG G). The PCR product was cloned into the TOPO-XL vector (Invitrogen). The resulting plasmid was then digested by HindIII and PstI (sites from the TOPO-XL vector). The fragment containing the operator/promoter region of the ccd_{O157} operon was inserted into pJL207 that was opened by the same enzymes.

The Topo-*folA*ccd_{O157} plasmid was constructed by amplifying the chromosomal region comprising the *folA* gene and the ccd_{O157} operon by PCR using *E. coli* O157:H7 chromosomal DNA (ATCC 700927) as a template and the following primers: 5′*folA* (5′CCC TCA TCC TAA TAA AGA GTG ACG) and 3′operon-ccd_{O157} (5′CGA ACC GGC ATA AGG ATT TGG GTG AGG G). The PCR product was cloned into the TOPO-XL vector (Invitrogen).

To construct the pKT-ccd_{O157} plasmid, the ccd_{O157} operon was amplified by PCR using *E. coli* O157:H7 chromosomal DNA (ATCC 700927) as a template and the following primers: 5′operon-ccd_{O157} (5′GAG ATT CTG GAG CGG CGG TAA TTT TG) and 3′operon-ccd_{O157} (5′CGA ACC GGC ATA AGG ATT TGG GTG AGG G). The PCR product was cloned into the TOPO-XL vector (Invitrogen). The resulting plasmid was digested by EcoRI and then inserted into the pKT279 plasmid digested by EcoRI.

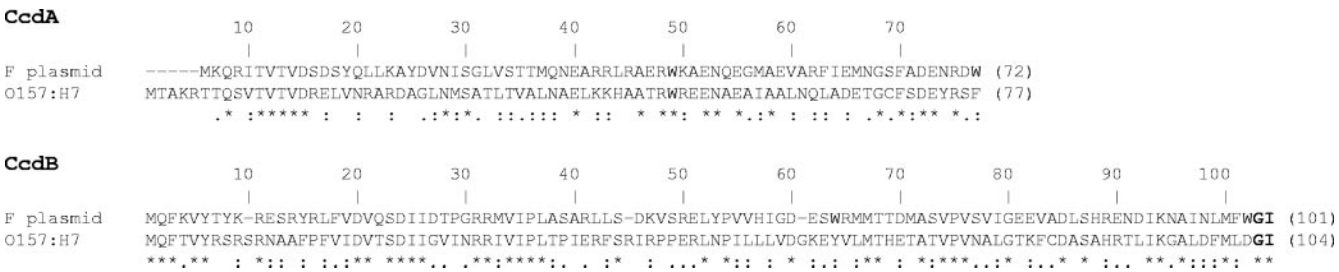

FIG. 1. Homology between the CcdA and CcdB proteins. Proteic sequences of the CcdA and CcdB proteins from the F plasmid and *E. coli* O157:H7 were aligned with the CLUSTALW program. Symbols: asterisk, identical amino acids; colon, strongly similar amino acids; period, weakly similar amino acids. The total number of amino acids for each protein is in parentheses. The G100 and I101 active sites are indicated in bold.

(iii) Postsegregational killing plasmids. To construct the pMLO-ccd_{O157} plasmid, the ccd_{O157} operon was amplified by PCR using *E. coli* O157:H7 chromosomal DNA (ATCC 700927) as a template and the following primers: 5′operon-ccd_{O157} (5′GAG ATT CTG GAG CGG CGG TAA TTT TG) and 3′operon-ccd_{O157} (5′CGA ACC GGC ATA AGG ATT TGG GTG AGG G). The PCR product was cloned into the TOPO-XL vector (Invitrogen). The resulting plasmid was then digested by HindIII and XbaI. The fragment containing the ccd_{O157} operon was blunted using the Klenow enzyme and then inserted into the pMLO59 plasmid digested by SmaI.

All the plasmids and the intermediate constructs that were constructed were sequenced.

Media. Luria-Bertani medium (LB) (37), Ceria 132 synthetic medium (CM) (25), and CM supplemented with 0.1% Casamino Acids (CCM) were used.

DNA manipulations. Transformations with appropriate plasmids were performed as described in reference 37, and most routine DNA manipulations were performed as described in reference 43.

Toxicity and antitoxicity assays. Strains carrying the toxin-expressing plasmids and/or the antitoxin-expressing plasmids were grown overnight (ON) at 37°C in CCM supplemented with glucose (0.4%) and the appropriate antibiotics. ON cultures were diluted in the same medium to an optical density (OD) at 600 nm of ~0.01 and grown at 37°C to an OD_{600} of ~0.1 to 0.2. The cultures were centrifuged at 4,000 rpm for 10 min at room temperature. The bacterial pellets were resuspended in CCM, prewarmed at 37°C, and supplemented with glycerol (0.4%) and the appropriate antibiotics. Arabinose was then added (0.25% or 1%), and the cultures were grown at 37°C. No IPTG (isopropyl-β-D-thiogalactopyranoside) was added. Samples were removed at 0, 10, 20, and 30 min, diluted in $MgSO_4$ (10 mM), and plated on CCM plates supplemented with glucose (0.4%) and the appropriate antibiotics. Plates were incubated ON at 37°C.

Postsegregational killing assay. O55:H7 and O55:H7Δccd_{O157} containing the pMLO59 vector and its derivatives were grown ON at 30°C in LB containing spectinomycin (100 μg/ml). ON cultures were centrifuged at 4,000 rpm for 10 min at room temperature and resuspended in LB. Cultures were then diluted 400-fold in LB and grown at 30°C and at 42°C. Cultures were diluted every 45 min to maintain an OD_{600} of ~0.1 to 0.2. Samples were removed at 0, 45, 90, 135, 180, 225, 270, and 315 min, diluted in $MgSO_4$ (10 mM), and plated on LB plates and on LB plates supplemented with spectinomycin. Plates were incubated ON at 30°C.

Promoter activity assay. CSH50/pJL-OPccd_F and CSH50/pJL-OPccd_{O157} containing the pKT279 vector or its derivatives were grown at 37°C in LB containing chloramphenicol (20 μg/ml) and tetracycline (15 μg/ml) to an OD_{600} of ~0.3. Samples were removed, and β-galactosidase assays were performed as described in reference 57.

Protein sample preparation, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and Western blot analysis. We transferred 1-ml samples from cultures to tubes containing 50 μl of cold 100% trichloroacetic acid. After centrifugation, pellets were washed twice with 500 μl of cold 100% acetone, air dried, and resuspended in SDS-gel loading buffer. Equal amounts of protein were separated on a 15% SDS-polyacrylamide gel electrophoresis gel and transferred to nitrocellulose filters. Filters were incubated with polyclonal anti-$CcdA_F$ antibodies. Immunoblots were developed by using horseradish peroxidase-conjugated goat anti-rabbit and enhanced chemiluminescence (Amersham).

Because of the low sensitivity of the anti-$CcdA_F$ antibodies against the $CcdA_{O157}$ protein, concentrations of protein extracts from strains producing $CcdA_{O157}$ were fivefold higher than those from strains producing $CcdA_F$ to reach comparable detection levels.

RESULTS

Diversity of the *folA-apaH* region in *E. coli* strains. Using TBLASTN, several ORFs coding for proteins nearly identical to the $CcdA_F$ and $CcdB_F$ proteins encoded by the F plasmid (83 to 100% identity) were found in virulence F-related plasmids (e.g., pO157 of *E. coli* O157:H7, pINV of *Shigella flexneri*, and pSLT of *Salmonella enterica* serovar Typhimurium). Interestingly, we found that ORFs coding for proteins with less identity (30% and 35% with $CcdA_F$ and $CcdB_F$, respectively) (Fig. 1) were present at the same chromosomal location in 5 of the 17 *E. coli* strains partially or totally sequenced (O157:H7 EDL933, O157:H7 VT-Sakai, O6:H1 CFT073, O6:H31 536, and O6:H31 F11). Note that all these strains are pathogenic. The ccd_F homolog is located in a 637-bp region, between the *folA* and the *apaH* genes, outside of any identifiable mobile genetic element. *folA* and *apaH* are metabolic genes encoding a dihydrofolate reductase and a diadenosine tetraphosphatase, respectively. This chromosomal homolog was named ccd_{O157}. The ccd_{O157} system is also present between *folA* and *apaH* in the UTI89 strain, although a frameshift (+1) mutation occurred in the ORF corresponding to $CcdB_{O157}$, leading most likely to an inactive protein. A 77-bp intergenic region between *folA* and *apaH* was detected in nine *E. coli* strains (K-12 MG1655, K-12 W3110, O103:H2 E22, O111:NM B171, O144 53638, O9 HS, O139:H28 E24377A, 101-1, and H10407). In two *E. coli* strains (O148:H28 B7 and O111:H9 E110019), a region of 1,434 bp was detected at the same location. We identified in this region a sequence without a start codon coding for 29 amino acids homologous to the C-terminal part of $CcdA_{O157}$. We did not find any sequence corresponding to $CcdB_{O157}$.

We studied the ccd_{O157} system of *E. coli* O157:H7, since O157:H7 isolates contain an F-related virulence plasmid (pO157) that carries the ccd_F system. Therefore, in these natural isolates, both *ccd* systems have been maintained throughout the evolution.

The *E. coli* O157:H7 chromosomal *ccd* system (ccd_{O157}) encodes a toxin-antitoxin gene pair. We tested the activity of $CcdA_{O157}$ and $CcdB_{O157}$ to determine whether the ccd_{O157} system encodes a toxin-antitoxin gene pair. $ccdB_F$ and $ccdB_{O157}$ were cloned in the pBAD33 vector under the control of the arabinose-inducible promoter pBAD, while $ccdA_F$ and $ccdA_{O157}$ were cloned in the compatible pKK223-3 vector under the control of the IPTG-inducible promoter pTac. *E. coli* K-12 strains contain-

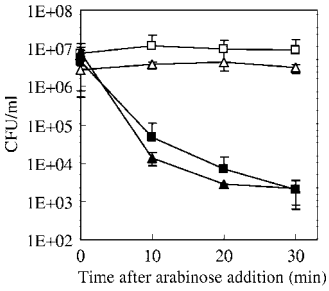

FIG. 2. The ccd_{O157} system carries a toxin-antitoxin gene pair. SG22622/pKK223-3/pBAD-$ccdB_F$ (filled squares), SG22622/pKK223-3/pBAD-$ccdB_{O157}$ (filled triangles), SG22622/pKK-$ccdA_F$/pBAD-$ccdB_F$ (open squares), and SG22622/pKK-$ccdA_{O157}$/pBAD-$ccdB_{O157}$ (open triangles) were grown as described in Materials and Methods. After the addition of 1% arabinose, serial dilutions of the cultures were plated without arabinose and incubated overnight at 37°C. Values correspond to the means of results of three independent experiments.

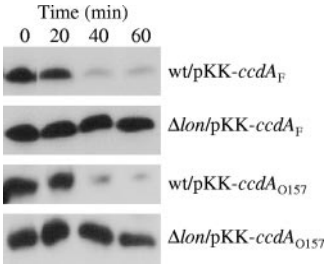

FIG. 3. Turnover of the $CcdA_F$ and $CcdA_{O157}$ antitoxins. SG226222 (wild type [wt]) and SG22623 (Δ*lon* mutant) carrying either the pKK-$ccdA_F$ or the pKK-$ccdA_{O157}$ plasmid were grown to early log phase in LB at 37°C. Spectinomycin was added (100 μg/ml) to block protein synthesis. Culture samples were removed at the times indicated in the figure. Protein extraction and Western blot analysis were performed as described in Materials and Methods.

ing the various constructs were grown exponentially, and $ccdB_F$ and $ccdB_{O157}$ transcription was induced at time zero by the addition of arabinose (Fig. 2). As observed for $CcdB_F$, the production of $CcdB_{O157}$ results in a dramatic decrease in viable counts (by about 2 logs) after 10 min of induction. Viability is not affected in the presence of the cognate antitoxins ($CcdA_F$ and $CcdA_{O157}$). This shows that the chromosomal ccd_{O157} system encodes functional toxin and antitoxin proteins.

$CcdB_F$ kills plasmid-free segregant bacteria by poisoning the DNA gyrase, an essential topoisomerase II (8, 9). To determine whether the $CcdB_{O157}$ toxin also targets the DNA gyrase, the pBAD-$ccdB_{O157}$ plasmid was transformed in a wild-type strain and in the isogenic strain carrying the $CcdB_F$-resistant mutation *gyrA462*. Table 2 shows that the transformation efficiency for the pBAD-$ccdB_F$ and pBAD-$ccdB_{O157}$ plasmids in the wild-type strain is very low in the presence of 1% arabinose ($<10^{-4}$), while it is comparable to that of the vector in the $CcdB_F$-resistant strain. This shows that the DNA gyrase is the cellular target of $CcdB_{O157}$. The key amino acids of the toxic active site of $CcdB_F$ have been previously identified as glycine100 and isoleucine101 (7). These two carboxy-terminal amino acids are conserved in $CcdB_{O157}$ (Fig. 1). We introduced the G100R or I101K mutations in $CcdB_{O157}$ and found that these mutations completely abolished the toxic activity of $CcdB_{O157}$ (data not shown), indicating that the active site of $CcdB_F$ is conserved in $CcdB_{O157}$.

$CcdA_F$ is unstable and degraded by the Lon ATP-dependent protease. Figure 3 shows that the half-life of $CcdA_{O157}$ in a wild-type strain is comparable to that of $CcdA_F$ (~30 min) and that both antitoxins are stabilized in a Δ*lon* strain. Thus, $CcdA_{O157}$ is a Lon substrate.

The putative promoter region of the ccd_{O157} system was cloned in a plasmid carrying a promoter-free *lacZ* gene (pJL-OPccd_{O157}). Table 3 shows that the promoter activity of the ccd_{O157} system is fivefold weaker than that of the ccd_F system. Regions of various lengths upstream and encompassing the 5′ end of the $ccdA_{O157}$ gene were tested, and their transcriptional activities are comparable to that of pJL-OPccd_{O157} (data not shown). We analyzed the autoregulation property of the ccd_{O157} system and found that its expression is autoregulated by $CcdA_{O157}$ and $CcdB_{O157}$ proteins expressed in *trans* (Table 3). However, the autoregulation appears to be less efficient than that of the ccd_F system (60% versus 90% of repression).

Interactions between the ccd_F and the ccd_{O157} systems. As the ccd_{O157} and the ccd_F systems coexist in *E. coli* O157:H7 isolates, we tested the cross-interactions between the components of the two systems. The ability of each antitoxin to counteract the toxic activity of its noncognate toxin was assayed in *E. coli* K-12. The expression of $CcdB_F$ and $CcdB_{O157}$ from the pBAD promoter results in a dramatic loss of viable counts. Basal expression of $CcdA_F$ from the pTac promoter in *trans* restores the viability of bacteria producing either $CcdB_{O157}$ or $CcdB_F$ (Fig. 4A). $CcdA_F$ is thus as able to efficiently counteract the toxic activity of $CcdB_{O157}$ as its cognate toxin. However, expression of $CcdA_{O157}$ from the pTac promoter is unable to counteract $CcdB_F$ toxicity (Fig. 4B), even under overproduction conditions (in the presence of 1 mM IPTG) (data not shown).

TABLE 2. Like $CcdB_F$, the $CcdB_{O157}$ toxin targets the DNA gyrase

Strain	Efficiency of transformation[a]		
	pBAD33	pBAD-$ccdB_F$	pBAD-$ccdB_{O157}$
SG22622	1.1	6.7×10^{-5}	7.1×10^{-5}
SG22622 *gyrA462*	1.1	0.9	1

[a] Efficiency of transformation was calculated as the ratio of the number of transformants obtained on 1% arabinose plates to the number of transformants obtained on plates without arabinose. This experiment was performed at least in triplicate.

TABLE 3. Promoter activity and autoregulation of the ccd_F and ccd_{O157} systems

OP::*lacZ* fusion and corresponding operon	β-Galactosidase sp act (Miller units) (mean ± SD)[a]
pJL-OPccd_F pKT279	233 ± 11.83 (1)
pJL-OPccd_F pKTccd_F	22 ± 0.76 (0.09)
pJL-OPccd_{O157} pKT279	46 ± 0.97 (1)
pJL-OPccd_{O157} pKTccd_{O157}	17 ± 1.96 (0.37)

[a] β-galactosidase-specific activity of the OP::*lacZ* fusions in the presence of the corresponding operon in *trans* was normalized to that obtained with the control vector (shown in parentheses). Values correspond to the means of results for three experiments ± standard deviations.

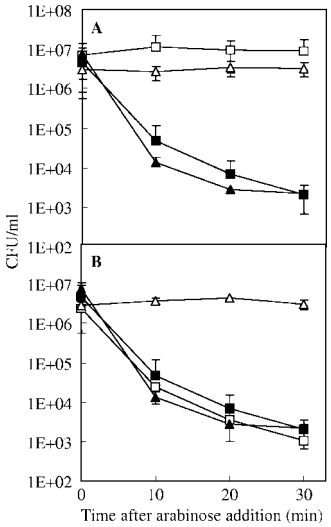

FIG. 4. Interactions between the ccd_F and the ccd_{O157} systems. All strains were grown as described in Materials and Methods and treated as described in the legend for Fig. 2. Values correspond to the means of results of three independent experiments. Data shown were obtained from the same experiments and are represented in two panels for clarity. (A) Ability of $CcdA_F$ to counteract the toxic activity of $CcdB_{O157}$ SG22622/pKK223-3/pBAD-$ccdB_F$ (filled squares), SG22622/pKK223-3/pBAD-$ccdB_{O157}$ (filled triangles), SG22622/pKK-$ccdA_F$/pBAD-$ccdB_F$ (open squares), and SG22622/pKK-$ccdA_F$/pBAD-$ccdB_{O157}$ (open triangles). (B) Ability of $CcdA_{O157}$ to counteract the toxic activity of $CcdB_F$ SG22622/pKK223-3/pBAD-$ccdB_F$ (filled squares), SG22622/pKK223-3/pBAD-$ccdB_{O157}$ (filled triangles), SG22622/pKK-$ccdA_{O157}$/pBAD-$ccdB_F$ (open squares), and SG22622/pKK-$ccdA_{O157}$/pBAD-$ccdB_{O157}$ (open triangles).

We also tested the cross-interactions between the ccd_F and the ccd_{O157} systems in an *E. coli* strain carrying the ccd_{O157} system in its chromosome. For that purpose, we could not use the O157:H7 strain, since it contains the pO157 plasmid carrying the ccd_F system.

Evolutionary analyses have shown that O55:H7 strains are genetically closely related to O157:H7 strains (55, 56), although they are devoid of the pO157 plasmid. We screened O55:H7 isolates by PCR using two sets of primers, one specific to ccd_{O157} and the other to ccd_F, and confirmed that these strains carry ccd_{O157} but lack ccd_F. DNA sequencing revealed that the ccd_{O157} system and its flanking regions are identical to those of O157:H7 (data not shown). A deletion of the ccd_{O157} system was constructed in O55:H7 (O55:H7Δccd_{O157}; see Materials and Methods).

Strains O55:H7 and O55:H7Δccd_{O157} were transformed with the pBAD-$ccdB_F$ and the pBAD-$ccdB_{O157}$ plasmids. The viabilities of both strains were measured upon expression of the $CcdB_F$ and $CcdB_{O157}$ toxins. Figure 5 shows that the viability of O55:H7 is only slightly affected after 30 min of $CcdB_{O157}$ induction, while it is largely reduced by that of $CcdB_F$ (by about 2 logs). This shows that $CcdA_{O157}$ is produced from its natural location at a basal level that is sufficient to at least partially counteract the toxic activity of $CcdB_{O157}$ produced in *trans*. These results also confirm that $CcdA_{O157}$ is unable to counteract $CcdB_F$ produced in *trans*. On the other hand, the viability of O55:H7Δccd_{O157} is drastically affected by the expression of both toxins (Fig. 5).

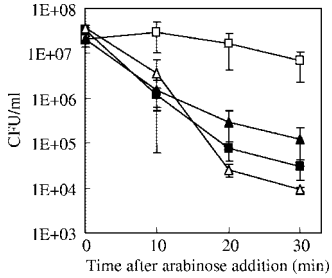

FIG. 5. Expression of the chromosomal ccd_{O157} system in the O55:H7 *E. coli* strain. O55:H7 (squares) and O55:H7Δccd_{O157} (triangles) containing pBAD-$ccdB_F$ (filled symbols) or pBAD-$ccdB_{O157}$ (open symbols) plasmids were grown as described in Materials and Methods. After addition of 0.25% arabinose, serial dilutions of the cultures were plated without arabinose and incubated ON at 37°C. Values correspond to the means of results for three independent experiments.

The ccd_F system is able to mediate postsegregational killing in the presence of its chromosomal homolog ccd_{O157}. The chromosomal $CcdA_{O157}$ antitoxin, whether expressed ectopically or in its natural context, is not able to counteract the plasmidic $CcdB_F$ toxin. Therefore, we tested whether the ccd_F system is able to mediate PSK in the presence of ccd_{O157}. The O55:H7 strain that carries the ccd_{O157} system in its chromosome was transformed with a conditionally replicating (thermosensitive) plasmid carrying the ccd_F system (pMLO59-ccd_F). Figure 6 shows that, after 180 min of culture at 42°C, the ability of O55:H7/pMLO59-ccd_F to form colonies decreases in comparison with that of the control strain O55:H7/pMLO59. It only slightly increases during the next 135 min of the experiment, showing that the loss of pMLO59-ccd_F mediates PSK. Thus, the ccd_F system is functional for plasmid stabilization in an *E. coli* strain carrying the ccd_{O157} system in its chromosome.

The ccd_{O157} system is unable to mediate postsegregational killing. TA systems located in mobile genetic elements such as plasmids (e.g., ccd_F and $relBE_{p307}$), prophages ($relBE_{K\text{-}12}$), and superintegrons (*higBA*) have been shown to be able to mediate PSK (17, 26, 27, 31). We tested the capacity of the chromosomal ccd_{O157} system to mediate PSK using the same system

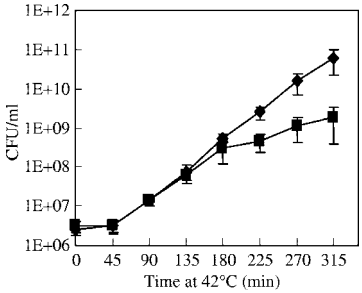

FIG. 6. The ccd_F system mediates postsegregational killing in the O55:H7 *E. coli* strain. O55:H7 containing the pMLO59 vector (diamond) and its derivative carrying the ccd_F system (squares) were grown as described in Materials and Methods. Exponential cultures in LB at 42°C were sampled at the times indicated in the figure. Surviving bacteria were scored as the number of CFU on LB plates at 30°C. Values correspond to the means of results for three independent experiments.

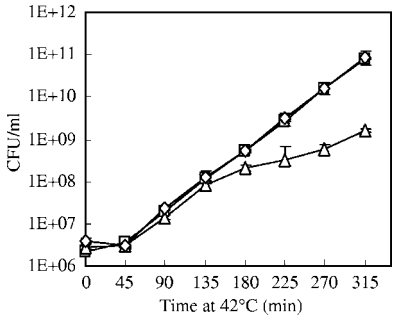

FIG. 7. The ccd_{O157} system is unable to mediate postsegregational killing. O55:H7Δccd_{O157} containing the pMLO59 vector (squares) and its derivatives carrying either the ccd_F (triangles) or the ccd_{O157} (diamonds) system were grown as described in Materials and Methods. Exponential cultures in LB at 42°C were sampled at the times indicated in the figure. Surviving bacteria were scored as the number of CFU on LB plates at 30°C. The values correspond to the means of results for three independent experiments.

described above. The O55:H7Δccd_{O157} strain was transformed with the pMLO59-ccd_F or the pMLO59-ccd_{O157} plasmid. As expected, the loss of pMLO59-ccd_F decreases the ability of the O55:H7Δccd_{O157} strain to form colonies after 180 min of culture at 42°C. On the contrary, the loss of pMLO59-ccd_{O157} does not affect the ability of the O55:H7Δccd_{O157} strain to form colonies, even after 315 min of culture at 42°C (Fig. 7). As a control, we confirmed that the loss of both plasmids is comparable to that of the pMLO59 vector control (data not shown).

DISCUSSION

TA systems are abundant in bacterial chromosomes and on mobile genetic elements such as plasmids, phages, and transposons (24). Computational analyses have grouped TA systems in families based on proteic sequence similarities and sequence profile analysis (5, 38). Members of a given family often coexist in the same bacteria. For instance, the *relBE* and the *vapBC* families are represented at 9 and 13 copies, respectively, in the chromosome of *Nitrosomonas europaea* (38). Situations in which plasmidic and chromosomal systems belonging to the same family are present in the same bacteria also exist. We have identified such a situation in the pathogenic *E. coli* O157:H7 strains, since they contain the plasmidic ccd_F system located on an F-derivative virulence plasmid (pO157) and a chromosomal homolog of this plasmidic system, the ccd_{O157} system.

The ccd_{O157} chromosomal system is located between the *folA* and *apaH* genes in the chromosomes of 5 of the 17 sequenced *E. coli* strains. This heterogeneous distribution among the *E. coli* species suggests that the ccd_{O157} system has been acquired by horizontal gene transfer. In the nine *E. coli* strains lacking it, a palindromic unit sequence is found in the *folA-apaH* intergenic region. Since palindromic units are specific targets for IS*1397* insertion (18), an attractive hypothesis is that a composite transposon carrying an ancestral *ccd* system and other genes may have hopped in this specific site. DNA rearrangements as well as coevolution with the host genome may have occurred (e.g., GC content of ccd_{O157} [49%] is close to that of the chromosome of O157:H7 [50%]), leading to the actual ccd_{O157} system. The *folA-apaH* intergenic region seems to be subject to insertion events, as a DNA segment containing an ORF coding for a hypothetical protein of unknown function and a sequence homologous to the C-terminal part of $CcdA_{O157}$ has been detected in 2 of the 17 *E. coli* strains (data not shown).

The chromosomal $relBE_{K\text{-}12}$ and *mazEF* systems of *E. coli* K-12 have been described as having a function in bacterial physiology (2, 14, 15, 16, 30, 47). However, each system seems to have a different function and/or a different function under different physiological conditions. *mazEF* is activated by numerous stresses and leads to cell death (22, 30), while activation of $relBE_{K\text{-}12}$ appears to be more specific to amino acid starvation and results in growth inhibition (15, 39). Although we do not yet have data involving the ccd_{O157} system in the *E. coli* physiology, we have shown that it is expressed in its natural context, i.e., in O55:H7 *E. coli* isolates. We have shown that, unlike the ccd_F system, the ccd_{O157} system is unable to mediate postsegregational killing. The weak promoter activity of ccd_{O157} in comparison with that of ccd_F, rather than the toxicity of $CcdB_{O157}$, is likely to be responsible for its inability to mediate PSK, since $CcdB_{O157}$, when expressed ectopically, causes a reduction in viability comparable to that of $CcdB_F$. A low transcriptional activity might reflect the adaptation of the ccd_{O157} system to its chromosomal location. It is likely that chromosomal TA systems might have to adapt to their hosts to be maintained throughout evolution. Therefore, their regulation might be more complex and integrated than that of plasmidic TA systems. For instance, the alarmone ppGpp regulates negatively the transcription of the *mazEF* system in *E. coli* K-12 (2).

The ccd_F and ccd_{O157} systems naturally coexist in O157:H7 isolates, as these strains contain an F-derivative virulence plasmid (pO157), which is ccd_F^+. Our cross-talk experiments have shown that the chromosomal $CcdA_{O157}$ antitoxin is not able to counteract the plasmidic $CcdB_F$ toxin, even under overproduction conditions, while the plasmidic $CcdA_F$ antitoxin is able to counteract the chromosomal $CcdB_{O157}$ toxin. Moreover, we have shown that the ccd_F system is able to mediate PSK in an *E. coli* O55:H7 strain harboring the ccd_{O157} system in its chromosome, showing that the ccd_F system is functional in the presence of its chromosomal counterpart. A similar observation was made for the *pem* (*parD*) TA system located on the R100 (R1) plasmid of *E. coli*. The two antitoxins of its chromosomal homologs (*mazEF* and *chpB*) are unable to counteract the toxicity of the plasmid-encoded toxin (PemK) (35), explaining how the *pem* system is able to stabilize plasmids in *E. coli* (51). However, mutants of the antitoxins of the *mazEF* and *chpB* systems are able to counteract PemK toxicity (44, 45), most likely due to an overproduction of the chromosomal antitoxins. In our experiments, overproduction of the chromosomal $CcdA_{O157}$ antitoxin does not counteract the plasmidic $CcdB_F$ toxin (data not shown). Thus, only plasmidic TA systems able to evade cross-interaction seem to coexist with their chromosomal homologs. This raises the hypothesis that chromosomal TA systems might serve as "exclusion" systems to protect bacteria from being loaded with an excess of exogenous DNA carrying identical TA systems (plasmids, transposons, phages) (18a, 41). a function somewhat reminiscent of that of

restriction-modification (RM) systems (for a review on RM, see reference 32). RM and TA systems share several properties. They are located in plasmids as well as in chromosomes, they seem to move from one location to another through horizontal gene transfer, and they are able to mediate PSK when cloned on an unstable plasmid. Further experiments are needed to test whether certain TA systems might promote the "exclusion" of incoming homologous TA systems.

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