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Chromosomal Toxin-Antitoxin Systems May Act as Antiaddiction Modules[▽]

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Toxin-antitoxin (TA) systems are widespread among bacterial chromosomes and mobile genetic elements. Although in plasmids TA systems have a clear role in their vertical inheritance by selectively killing plasmid-free daughter cells (postsegregational killing or addiction phenomenon), the physiological role of chromosomally encoded ones remains under debate. The assumption that chromosomally encoded TA systems are part of stress response networks and/or programmed cell death machinery has been called into question recently by the observation that none of the five canonical chromosomally encoded TA systems in the *Escherichia coli* chromosome seem to confer any selective advantage under stressful conditions (V. Tsilibaris, G. Maenhaut-Michel, N. Mine, and L. Van Melderen, J. Bacteriol. 189:6101–6108, 2007). Their prevalence in bacterial chromosomes indicates that they might have been acquired through horizontal gene transfer. Once integrated in chromosomes, they might in turn interfere with their homologues encoded by mobile genetic elements. In this work, we show that the chromosomally encoded *Erwinia chrysanthemi ccd* (control of cell death) (ccd_{Ech}) system indeed protects the cell against postsegregational killing mediated by its F-plasmid *ccd* (ccd_F) homologue. Moreover, competition experiments have shown that this system confers a fitness advantage under postsegregational conditions mediated by the ccd_F system. We propose that ccd_{Ech} acts as an antiaddiction module and, more generally, that the integration of TA systems in bacterial chromosomes could drive the evolution of plasmid-encoded ones and select toxins that are no longer recognized by the antiaddiction module.

Genes are often expected to be an integral part of genetic circuits in an organism and to possess a dedicated role in cellular functioning or adaptation. However, it is also possible that the presence of some genes may rather be the result of past evolutionary processes, implying that they might be devoid of any current physiological role on their own. We propose this view of the chromosomally encoded toxin-antitoxin (TA) systems, which are surprisingly abundant in bacterial chromosomes (24, 32). TA systems were originally discovered on low-copy-number plasmids. On such plasmids, they contribute to plasmid maintenance in growing bacterial populations by selectively eliminating daughter bacteria that do not receive a plasmid copy (postsegregational killing [PSK]) (15). The molecular mechanism underlying PSK is based on a differential stability of the toxin and antitoxin proteins encoded by the TA operon (39, 40). The toxin is a stable protein whose toxic activity is counteracted by the unstable antitoxin protein. In plasmid-free daughter bacteria, since the antitoxin is degraded and not replenished, the toxin is released from the TA complex and is able to exert its lethal activity. Yarmolinsky proposed renaming the plasmid-encoded TA systems as addiction modules, since the progeny of a bacterial cell containing a plasmid-encoded system become dependent on the presence of the otherwise dispensable plasmid (42).

Numerous homologues of plasmid-encoded TA systems are found in bacterial chromosomes. Many of these systems are associated with mobile genetic elements that constitute genomic islands, suggesting that horizontal gene transfer contributes to their dissemination (24). The biological function(s) of the chromosomally encoded TA systems has been an area of intense debate for several years (1, 20, 25, 38). Based on the work on the *mazEF* and *relBE* systems of *Escherichia coli*, the general idea that has emerged is that TA systems are involved in general stress management. *mazEF* has been shown to be responsible for programmed cell death under a wide variety of seemingly unrelated stressful conditions (e.g., short-term antibiotic treatments, high temperature, and oxidative shock) by the group of Engelberg-Kulka (14, 18, 31). However, chromosomally encoded TA system-dependent programmed cell death was not observed in work by other groups (5, 7, 22, 25, 26, 33, 34) and was recently ruled out by our group (38). The other mainstream hypothesis is that amino acid starvation induces chromosomally encoded TA system-dependent bacteriostasis. This was proposed by the group of Gerdes based mainly on the study of the *relBE* and *mazEF* systems (6, 7, 25). In this model, the assumption is that bacteriostasis is an advantage for the bacteria during starvation. However, in competition experiments, no selective advantage conferred by chromosomally encoded TA systems during either nutrient starvation (or other stress conditions) or the poststress recovery phase could be detected (38). Recently published work demonstrated that mRNA cleavage induced by amino acid starvation occurs independently of the five chromosomally encoded TA systems (the toxins of which are endoribonucleases) in *E. coli*, reinforcing the idea that the TA systems are not involved in stasis induced by starvation (19).

In this paper, we propose to reconsider the function of

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant description[a]	Reference or source
Strains		
MG1655	Wild-type *E. coli* K-12	12
NM529	MG1655 Δ*ara leu*::*Tet* Δ*lac*	From N. Majdalani
NM532	MG1655 Δ*ara* Δ*lac lacI*^{q}	From N. Majdalani
MS7	MG1655 ccd_{O157}^+	This work
MS8	MG1655 ccd_{Ech}^+	This work
MS10	MG1655 ccd_{Ech}^+ Δ*ara leu*::*Tet*	This work
MS20	MG1655 Δ*ara leu*::*Tet*	This work
NC397	W3110 *gal490* pglΔ8 attL int-N* pL *cI857*Δ (*cro-bioA*) *bio*Δ *lacI* <*kan* – T1 – *cat* – *sacB*> *plac*	From N. Majdalani
MS2	MG1655 *cat-sacB*	This work
SG22622	MC4100 *cpsB*::*lacZ* Δ*ara malP*::*lacI*^{q}	From S. Gottesman
SG22622*gyrA*$_{462}$	SG22622 *gyrA462 zei*::Tn*10*	Our laboratory
Plasmids		
pBAD33	p15A, Cm^r, pBAD promoter	13
pBAD-$ccdB_F$	$ccdB_F$ under the control of the pBAD promoter	41
pBAD-$ccdB_{Ech}$	$ccdB_{Ech}$ under the control of the pBAD promoter	This work
pKK223-3	ColE1, Amp^r, pTac promoter	4
pKK-$ccdA_F$ (pULB2709)	$ccdA_F$ under the control of the pTac promoter	29
pKK-$ccdA_{Ech}$	$ccdA_{Ech}$ under the control of the pTac promoter	This work
pMLO59	pGB2 ts derivative, Spec^r	From M. Labocka
pMLO-ccd_F (pULB2710)	pMLO59 containing the ccd_F operon	40
pMLO-ccd_{Ech}	pMLO59 containing the ccd_{Ech} operon	This work

[a] Cm^r, chloramphenicol resistant; Amp^r, ampicillin resistant; Spec^r, spectinomycin resistant; ts, temperature-sensitive.

chromosomally encoded TA systems, taking into account the interactions between plasmid-encoded and chromosomally encoded homologous TA systems. We tested the possibility that chromosomally encoded TA systems may act as antiaddiction modules such that they might protect the host bacteria against PSK mediated by their plasmid-encoded counterparts. The prediction is that their presence might confer a selective advantage under PSK conditions.

MATERIALS AND METHODS

Strains, plasmids, and media. Plasmids and strains used in this work are listed in Table 1. The sequences of the primers used in this work are listed in Table 2.

Construction of plasmids. (i) Expression plasmids. (a) pBAD-$ccdB_{Ech}$ plasmid. The *Erwinia chrysanthemi ccd* (control of cell *d*eath) ($ccdB_{Ech}$) gene was amplified by PCR using *E. chrysanthemi* 3937 chromosomal DNA as the template and the *ccdBEch-for* and *ccdBEch-rev* primers. The PCR product was cloned into the TOPO-XL vector (Invitrogen). The resulting plasmid was then digested using XbaI and PstI. The fragment containing $ccdB_{Ech}$ was inserted into the pBAD33 vector cut with the same enzymes.

(b) pKK-$ccdA_{Ech}$ plasmid. The $ccdA_{Ech}$ gene was amplified by PCR using *E. chrysanthemi* 3937 chromosomal DNA as the template and the *ccdAEch-for* and *ccdAEch-rev* primers. The PCR product was cloned into the TOPO-XL vector (Invitrogen). The resulting plasmid was then digested using EcoRI and PstI. The fragment containing $ccdA_{Ech}$ was inserted into the pKK223-3 vector cut with the same enzymes.

(ii) PSK plasmid (pMLO-ccd_{Ech} plasmid). The ccd_{Ech} operon was amplified by PCR using *E. chrysanthemi* 3937 chromosomal DNA as the template and the *ccdEch-for* and *ccdEch-rev* primers. The PCR product was cloned into the TOPO-XL vector (Invitrogen). The resulting plasmid was then digested by EcoRI and inserted into the PMLO59 vector cut using the same enzyme.

Construction of bacterial strains. (i) ccd_{Ech} and ccd_{O157} strains. The *cat-sacB* cassette was amplified by PCR using genomic DNA of the NC397 strain as the template and the *catsac-for* and *catsac-rev* primers. The resulting amplification product contained homologous sequences to the insertion point, i.e., the *folA-apaH* intergenic region at its 5′ end and homologous sequence of the *apaH* 5′ end at its 3′ end. The product was inserted in the *E. coli* MG1655 chromosome by use of the method described in reference 8. Chloramphenicol-resistant recombinants were selected and screened for sucrose sensitivity. The recombinant MG1655 *cat-sacB* strain was called MS2. The ccd_{Ech} and *E. coli* O157:H7 *ccd* (ccd_{O157}) TA systems were then introduced at that same location. The systems were amplified by PCR using the *IccdEch-for* and *IccdEch-rev* or the *IccdO157-for* and *IccdO157-rev* primers, and *E. chrysanthemi* 3937 or *E. coli* O157:H7 genomic DNA, respectively, as the template. The resulting amplification products containing homologous sequences to the flanking regions of the *cat-sacB*

TABLE 2. Sequences of the primers used in this work

Primer	Sequence (5′ to 3′)
ccdAEch-for	GAATTCATGAAACACCGCGTC
ccdAEch-rev	CTGCAGTCACCAGTTCCTG
ccdBEch-for	TCTAGAAGGAGGTGATCATGCAATTCATT
ccdBEch-rev	CTGCAGTCAAATCCCCCAA
ccdEch-for	CTATATCGTGCCCAACCCGC
ccdEch-rev	GCGAACACTCTGACTCCCCC
catsac-for	TCGCGTCTTATCCGGCCTTCCTATATCAGGCTGTGTTTAAAAAATGAGACGTTGATCGGC
catsac-rev	CGTCGAACCGGCATAAGGATTTGGGCGAAGCGGCGGCGTCATCAAAGGGAAAACTGTCCAT
IccdEch-for	CCGGTCGCGTCTTATCCGGCCTTCCTATATCAGGCTGTGTTCAATAGCGTGAAATACGCCAT
IccdEch-rev	CGTCGAACCGGCATAAGGATTTGGGCGAAGCGGCGGCGTCTTAAATCCCCCAAAACATAAGAT
IccdO157-for	CCGGTCGCGTCTTATCCGGCCTTCCTATATCAGGCTGTGTGGTATTCAGCGAATTCCACG
IccdO157-rev	CGTCGAACCGGCATAAGGATTTGGGCGAAGCGGCGGCGTCTTAAATCCCGTCGAGCATAA

cassette were inserted in the chromosome of MS2. Sucrose-resistant strains were selected. The ccd_{Ech} (MS8) and ccd_{O157} (MS7) strains were obtained and the inserted sequences and flanking regions were sequenced.

(ii) MG1655 Δ*ara* (MS20) and ccd_{Ech} Δ*ara* (MS10) strains. The MG1655 Δ*ara* and ccd_{Ech} Δ*ara* strains were constructed by transducing Δ*ara leu*::Tet (Tet indicates tetracycline resistance) from the MG1655 derivate NM529 by use of P1*vir* as described in reference 21.

Media. Luria-Bertani liquid and agar medium (LB) (Invitrogen) and MacConkey agar medium (Difco) were used.

DNA manipulations. Transformations with appropriate plasmids were performed according to reference 21, and most routine DNA manipulations were done as described in reference 30.

Toxicity and antitoxicity assay. Strains carrying the pKK223-3 vector or its derivatives expressing either $ccdA_F$ (pKK-$ccdA_F$) or $ccdA_{Ech}$ (pKK-$ccdA_{Ech}$) were transformed with the pBAD33 vector or its pBAD-$ccdB_F$ and pBAD-$ccdB_{Ech}$ derivatives. Transformation mixtures were plated on LB with appropriate antibiotics (ampicillin at 500 μg/ml and chloramphenicol at 20 μg/ml) with or without arabinose (1%). Plates were incubated overnight (ON) at 37°C. The 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.

PSK assay. The MG1655 strain or its ccd_{Ech} (MS8) and ccd_{O157} (MS7) derivatives containing the pMLO59 vector or its derivates encoding the F-plasmid *ccd* (ccd_F) system (pMLO-ccd_F) were grown ON at 30°C in LB liquid medium containing spectinomycin (100 μg/ml). ON cultures were diluted 800-fold in LB prewarmed to 42°C. Tenfold dilutions were performed after 2 and 3 h to maintain the cultures in log phase. Every 60 min, samples were plated on LB agar plates with or without spectinomycin (50 μg/ml).

Competition experiments. ON cultures of the MG1655 strain or its Δ*ara* derivative (MS20) containing either the pMLO59 vector or the pMLO-ccd_F plasmid were mixed at a 1:1 ratio with the ccd_{Ech} strain (MS8) or its Δ*ara* derivative (MS10) containing the pMLO-ccd_F plasmid. Cocultures were diluted 400-fold in prewarmed LB medium. Competition experiments were carried out as the PSK experiments described above, except that dilutions of the cultures were plated on MacConkey agar plates containing 1% arabinose with or without spectinomycin (50 μg/ml) to distinguish between ara^+ and Δ*ara* strains.

Phylogenetic analysis. Chromosomally encoded and plasmid-encoded homologues of the toxin and antitoxin proteins belonging to the ccd_F and ccd_{O157} systems were selected using TBlastN on complete sequenced genomes of gammaproteobacteria. Twenty-eight CcdA and 24 CcdB homologue open reading frames (ORFs) with a maximum E value of 10^{-6} were considered. Phylogenetic analyses were carried using the neighbor-joining method (28). The optimal trees for CcdA and CcdB homologues with the sums of branch length of 4.1 and 4.0, respectively, are shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (10). The tree is drawn to scale, with branch lengths in units the same as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the matrix-based method of Dayhoff (9) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the data set (complete deletion option). There were a total of 72 positions in the final data set of CcdA homologues and a total of 70 positions in the final data set of CcdB homologues. Phylogenetic analyses were conducted by MEGA4 (36).

RESULTS

The chromosomally encoded ccd_{Ech} system encodes a TA gene pair. To test the antiaddiction hypothesis, chromosomally encoded ORFs closely related to that of the ccd_F system of the *E. coli* F plasmid were selected using the TBlastN software. Two adjacent ORFs encoded in the chromosome of *E. chrysanthemi* 3937 appeared as candidates, since they presented 65% and 61% identity with the $CcdA_F$ antitoxin and the $CcdB_F$ toxin, respectively (data not shown). They were named $ccdA_{Ech}$ and $ccdB_{Ech}$, respectively. The effect of their ectopic expression on bacterial viability was assessed in the *E. coli* strain MG1655. Figure 1A shows that the $ccdB_{Ech}$ gene encodes a functional toxin whose toxic activity is counteracted by coexpression of the $ccdA_{Ech}$ gene. Like its $CcdB_F$ and $CcdB_{O157}$ counterparts

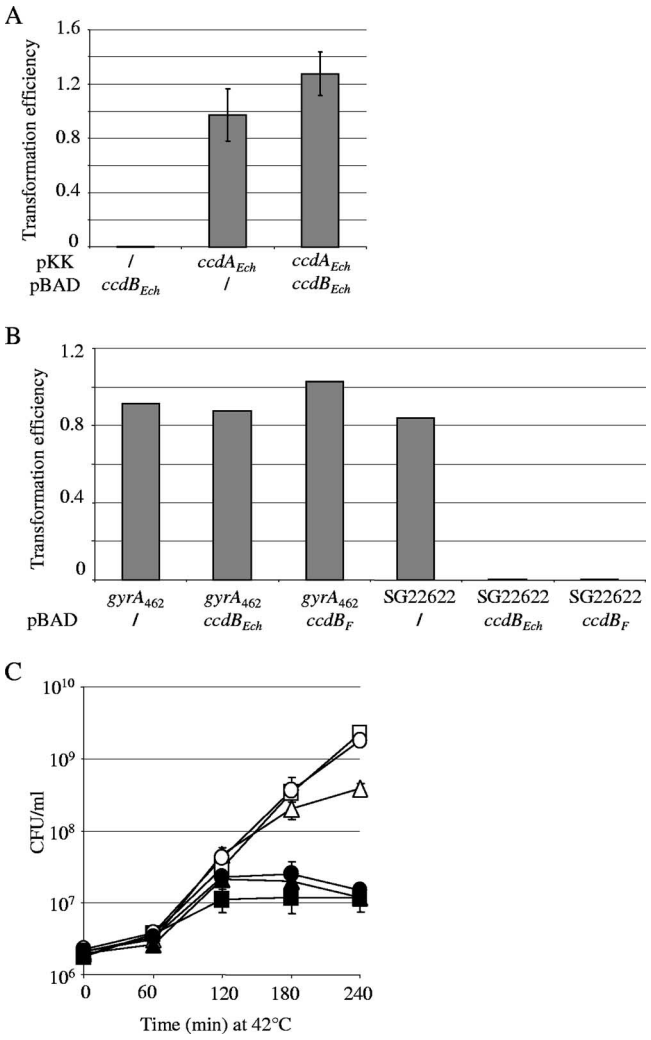

FIG. 1. Characterization of the chromosomally encoded ccd_{Ech} system. (A) ccd_{Ech} encodes functional antitoxin and toxin proteins. MG1655 strains carrying either the pKK223-3 vector (/) or its pKK-$ccdA_{Ech}$ derivative ($ccdA_{Ech}$) were transformed with the compatible pBAD33 vector (/) or its pBAD-$ccdB_{Ech}$ derivative ($ccdB_{Ech}$). The efficiency of transformation was calculated as the ratio of the number of transformants obtained on arabinose plates to the number of transformants obtained on plates without arabinose. Values correspond to the mean of three independent experiments. (B) The $CcdB_{Ech}$ toxin targets the GyrA subunit of DNA gyrase. The SG22622 strain and its $gyrA_{462}$ derivative were transformed with the pBAD33 vector (/) or its pBAD33-$ccdB_{Ech}$ ($ccdB_{Ech}$) and pBAD33-$ccdB_F$ ($ccdB_F$) derivatives. The 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. (C) The ccd_{Ech} system is unable to mediate PSK. MG1655 strains carrying either pMLO59 (squares) or its derivatives encoding ccd_{Ech} (pMLO-ccd_{Ech}; circles) and ccd_F (pMLO-ccd_F; triangles) were grown at 42°C. Viability (CFU/ml) was monitored for 240 min by plating serial dilutions on LB agar plates with (filled symbols) or without (open symbols) spectinomycin. Values correspond to the mean of three independent experiments.

(3, 41), $CcdB_{Ech}$ targets the DNA gyrase, since a $CcdB_F$-resistant mutant ($GyrA_{462}$ mutant) is also resistant to $CcdB_{Ech}$ (Fig. 1B). As observed for the chromosomally encoded ccd_{O157} system from the *E. coli* O157:H7 strain (41), ccd_{Ech} is unable to

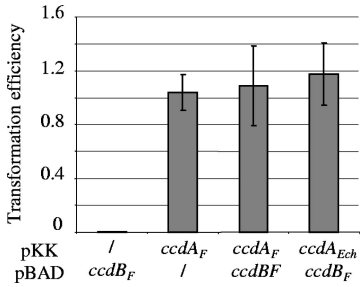

FIG. 2. $CcdA_{Ech}$ antagonizes $CcdB_F$ toxic activity. MG1655 strains containing either the pKK223-3 vector (/) or pKK-$ccdA_{Ech}$ ($ccdA_{Ech}$) and pKK-$cidA_F$ ($ccdA_F$) were transformed with the pBAD33 vector (/) or its pBAD-$ccdB_F$ derivative ($ccdB_F$). The efficiency of transformation was calculated as the ratio of the number of transformants obtained on 1% arabinose plates to the number of transformants obtained on plates without arabinose. Values correspond to the mean of three independent experiments.

mediate PSK when cloned in the replication-thermosensitive pMLO59 vector; this contrasts with ccd_F, which leads to PSK. Figure 1C shows that the viability of MG1655 is not affected by the loss of the pMLO-ccd_{Ech}, while the loss of pMLO-ccd_F leads to a 1-log loss of viability, as previously described in references 16, 40, and 41. This experiment was conducted for longer time without gaining any effect in PSK (data not shown).

The chromosomally encoded ccd_{Ech} system acts as an antiaddiction module. Our definition of an antiaddiction module would be genes that help to protect the cell from the loss of a plasmid carrying an addiction system. To evaluate whether ccd_{Ech} might constitute an antiaddiction module for a plasmid carrying ccd_F, we tested first the ability of $CcdA_{Ech}$ to inhibit the toxic activity of the F-plasmid-encoded $CcdB_F$ toxin. A pBAD plasmid carrying the $ccdB_F$ toxin gene cannot be successfully transformed into cells unless they also express the antitoxin to this toxin (Fig. 2, compare column 1 and column 3). Ectopic overexpression of $CcdA_{Ech}$ is able to inhibit the toxic activity of $CcdB_F$ as efficiently as the $CcdA_F$ antitoxin (Fig. 2, compare column 4 to column 3).

We then tested whether the ccd_{Ech} system could interfere with ccd_F-mediated PSK. For this purpose, the ccd_{Ech} system was inserted in the MG1655 chromosome. As a control, we chose to insert the ccd_{O157} system, which naturally occurs in *E. coli* isolates such as O157:H7 and O55:H7 and was previously shown to be unable to interfere with ccd_F-mediated PSK (41). Both systems were inserted in MG1655 between the *folA* and *apaH* genes (at the natural location of the ccd_{O157} system in O157:H7 and O55:H7 strains), giving rise to the ccd_{Ech} (MS8) and ccd_{O157} (MS7) strains, respectively (see Materials and Methods). The loss of pMLO-ccd_F affects the viability of the MG1655 and ccd_{O157} strains similarly, with a decrease of viability of ~1 log (Fig. 3A and B). However, the presence of ccd_{Ech} in the ccd_{Ech} strain prevents this loss in viability and therefore protects the cell against ccd_F-mediated PSK (Fig. 3C). These data show that ccd_{Ech} acts as an antiaddiction module with respect to the plasmid-encoded ccd_F system.

ccd_{Ech}-dependent gain of fitness under ccd_F-mediated PSK conditions. To test whether the ccd_{Ech} conferred a selective advantage under our experimental conditions, competition experiments between the MG1655 and ccd_{Ech} strains were carried out. A deletion of the arabinose operon (Δ*ara*) was introduced in both strains to allow their discrimination during competition experiments. Figure 4 shows that the strain carrying the ccd_{Ech} module had an advantage over a strain that did not carry it (relative fitness, 1.25 [columns 2 and 6]) only when both carried the pMLO-ccd_F plasmid as well (compare column 2 to column 1 and column 6 to column 5). The fitness of the ccd_{Ech}/pMLO-ccd_F and MG1655/pMLO-ccd_F strains was similar when they were cocultivated with their Δ*ara* derivatives, showing that the *ara* deletion does not interfere with the growth rate (columns 3 and 4). These results show that the ccd_{Ech} system confers a selective advantage of 25% in ccd_F-mediated PSK conditions. This gain in fitness may not be more dramatic because the ccd_F system is not a very efficient stabilization system (10-fold stabilization) (Fig. 1B) (16, 40, 41).

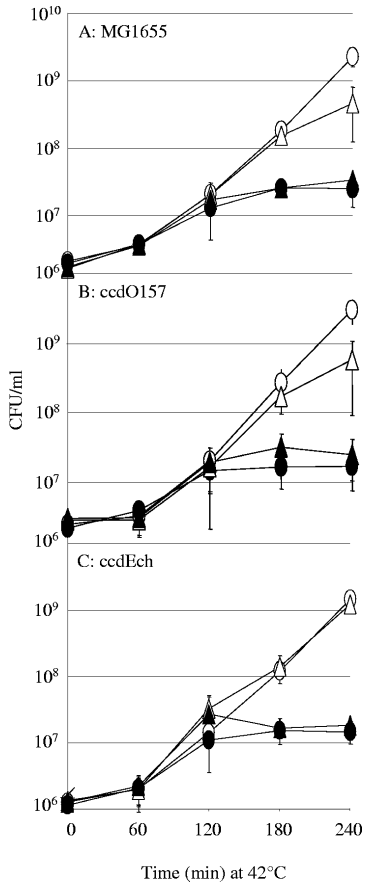

FIG. 3. ccd_{Ech} is an antiaddiction module. The MG1655 (A), ccd_{O157} (B), and ccd_{Ech} (C) strains containing either the pMLO59 replication-thermosensitive plasmid (spectinomycin resistance) (circles) or its derivative encoding the ccd_F system (pMLO-ccd_F; triangles) were grown at 42°C. Viability (CFU/ml) was monitored for 240 min by plating serial dilutions on LB agar plates with (filled symbols) or without (open symbols) spectinomycin. Values correspond to the mean of three independent experiments.

DISCUSSION

The wide occurrence of TA systems in bacterial chromosomes is quite striking (24, 32). In bacterial species such as

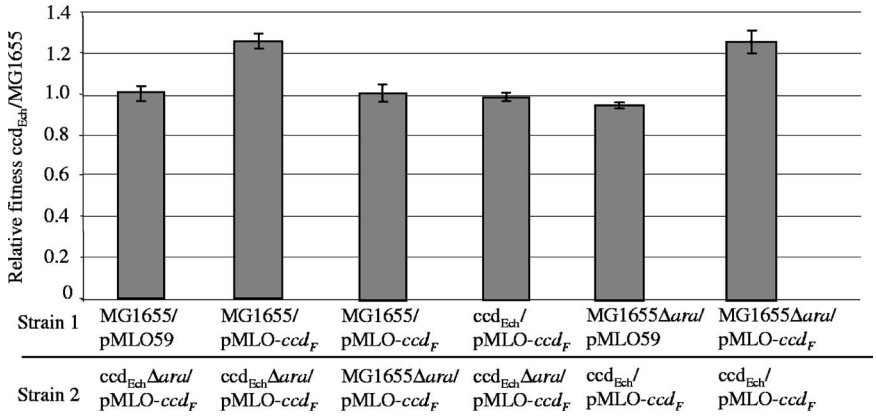

FIG. 4. ccd_{Ech} increases fitness under the PSK condition. Competition experiments between the MG1655 strain containing either the pMLO59 vector (column 1) or the pMLO-ccd_F plasmid (column 2) and the ccd_{Ech} Δ*ara* strain containing the pMLO-ccd_F plasmid were carried out. As controls, (i) each strain was cocultivated with its Δ*ara* derivative, i.e., MG1655/pMLO-ccd_F in competition with MG1655 Δ*ara*/pMLO-ccd_F (column 3) and ccd_{Ech}/pMLO-ccd_F in competition with ccd_{Ech}Δ*ara*/pMLO-ccd_F (column 4); and (ii) the "mirror" competition experiment was carried out with the MG1655 Δ*ara* strain containing either the pMLO59 vector (column 5) or the pMLO-ccd_F plasmid (column 6) and the ccd_{Ech} strain containing the pMLO-ccd_F plasmid. Cultures were mixed at a 1:1 ratio and cocultures grown under conditions similar to those used for the PSK experiments (see Materials and Methods). Viability (CFU/ml) was monitored for 240 min by plating serial dilutions on MacConkey arabinose (1%) agar plates. Relative fitness was calculated as described in reference 11 and represents the advantage of the ccd_{Ech} strain relative to MG1655. Values correspond to the mean of three independent experiments.

Vibrio cholerae and *Nitrosomonas europaea*, they tend to cluster in large superintegron structures (24, 27), and recent data have shown that they stabilize large genomic regions by reducing large-scale deletion (33). Thus, hypotheses regarding the biological functions of chromosomally encoded TA systems other than the stress management ones are emerging. Moreover, recent data questioned this last biological role, at least in the *E. coli* model and under the experimental conditions tested (38). However, as TA systems are extremely diversified, one cannot exclude the possibility that some are integrated in genetic circuits, notably in specific bacterial species which undergo development, as was recently described for *Myxococcus xanthus* (23).

The abundance of chromosomally encoded TA systems has been proposed to be correlated to the bacterial lifestyle, since Gerdes and colleagues have observed that TA systems are more abundant in free-living bacterial species chromosomes than in obligate intracellular bacteria (24). This observation has been interpreted in light of the stress management hypothesis, i.e., free-living bacteria are subjected to ever-changing environments and have to cope with a multitude of different stresses (24). An alternative view is that the occurrence of TA systems in bacterial chromosomes is correlated to the rate of horizontal gene transfer. Once integrated in chromosomes, these systems might in turn interfere with their mobile genetic element-encoded homologues. This raises the possibility that chromosomally encoded TA systems might serve as a defense mechanism against invading DNA (plasmids or phages) in a way that was proposed by Mazel and his collaborators (27) and reminiscent of restriction-modification systems and CRISPR regions (2, 17, 37). It was notably shown by the group of Kobayashi that solitary chromosomally encoded methylase can protect the bacterial host against PSK mediated by a plasmid-encoded restriction-modification system (35). The data presented in the present work demonstrate that the chromosomally encoded ccd_{Ech} system is indeed able to interfere with its close ccd_F homologue and thereby to protect the cell against ccd_F-mediated PSK. Competition experiments under PSK conditions showed that a gain in fitness is conferred by ccd_{Ech}. These results lead us to propose that newly integrated chromosomally encoded TA systems could act de facto as antiaddiction modules due to their new location and their high similarity with plasmid-encoded homologues. The positive effect of antiaddiction modules on host fitness could favor their fixation within populations subjected to the lethal activity of the plasmid-encoded homologues. In turn, this could drive the directional selection of natural variants of these plasmid-encoded systems, in which the toxin is no longer recognized by the antiaddiction module. This evolution of plasmid-encoded TA systems toward a group distinct enough to be resistant to chromosomally encoded antitoxin is supported by our phylogenetic analysis of the *ccd* systems found in gammaproteobacteria. Figure 5 shows that plasmid-encoded *ccd* systems form a monophyletic group (α1) that is distantly related to the chromosomally encoded one (β). Interestingly, the ccd_{Ech} antiaddiction module belongs to the chromosomally encoded α2 group, which is closely related to the α1 plasmid-encoded group. Coevolution of TA systems might have led to the divergence of chromosomally encoded TA systems and their plasmid-encoded homologues into two distinct groups which might allow for the "harmonious" coexistence of both types of systems, as observed for the *E. coli* O157:H7 strain (41). In this case, the chromosomally encoded ccd_{O157} system does not interfere with PSK mediated by the ccd_F system harbored by the F-related pO157 virulence plasmid (41). This situation might represent the result of evolutionary events that have led to the selection of plasmid-encoded TA systems having the capacity to avoid any functional interference caused by their chromosomal homologues. Therefore, some chromosomally encoded TA systems might simply be remnants of past evolutionary events and could be devoid of any current physiological role. Frameshift mutations found in the CcdB ORFs might indicate the decay of these systems. A more global understand-

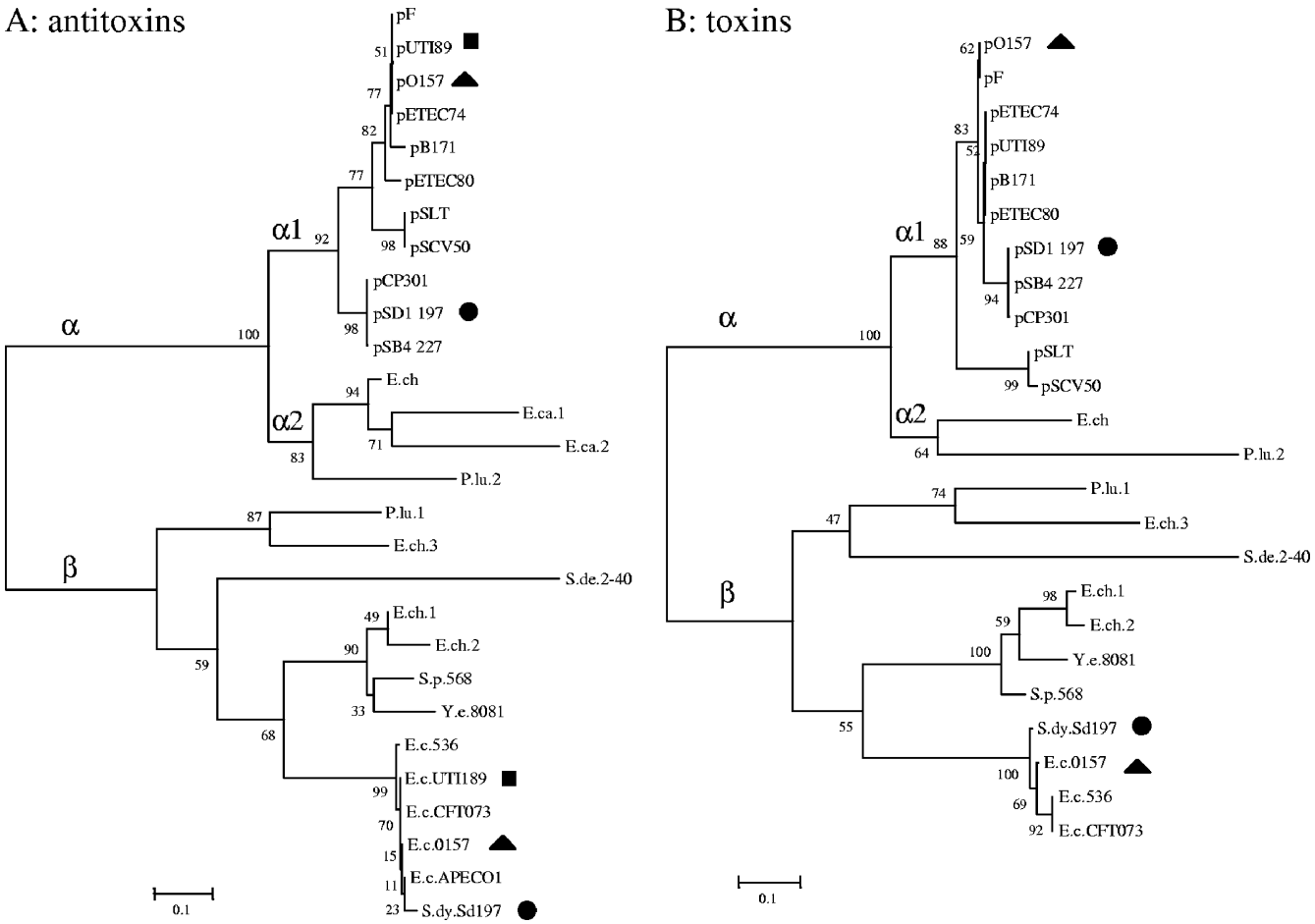

FIG. 5. Phylogenetic analysis of the ccd_F homologues. By use of the TBlastN software, 27 and 23 homologues of $CcdA_F$ and $CcdB_F$, respectively, were selected in the complete sequenced genomes of gammaproteobacteria. Phylogenetic relationships were inferred by analyzing amino acids sequences of antitoxins and toxins as described in Materials and Methods. Names of bacterial species are abbreviated as follows: *E. chrysanthemi* 3937 (E.ch, E.ch.1, E.ch.2, and E.ch.3), *E. carotovora* subsp. *atroseptica* SCRI1043 (E.ca.1 and E.ca.2), *Photorabdus luminescens* subsp. *laumondii* TTO1 (P.lu), *E. coli* O157:H7 (E.c.O157), *E. coli* UTI189 (E.c.UTI189), *E. coli* CFT073 (E.c.CFT073), *E. coli* 536 (E.c.536), *E. coli* APEC01 (E.c.APEC01), *Shigella dysenteriae* Sd197 (S.dy.Sd197), *Saccharophagus degradans* 2-40 (S.de.2-40), *Yersinia enterocolitica* subsp. *enterocolitica* 8081 (Y.e.8081), and *Serratia proteamaculans* 568 (S.p.568). Naturally coexisting plasmid-encoded TA systems and chromosomally encoded TA systems are indicated by symbols as follows: O157:H7 contains the pO157 plasmid (triangles), UTI189 contains the pUTI89 plasmid (squares), and Sd197 contains the pSD1 197 plasmid (circles). Note that for E.ca.1, E.ca.2, E.c.APEC01, and E.c.UTI189, CcdA ORFs were detected, while cognate CcdB-encoding ORFs either were not (E.ca.1) or contained frameshift mutations (E.ca.2, E.c.APEC01, and E.c.UTI189) that are predicted to inactivate CcdB.

ing of TA system evolution, distribution, and mobility will certainly help to decipher the biological meaning of their presence in bacterial genomes.

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