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A Role for Single-Stranded Exonucleases in the Use of DNA as a Nutrient[▽]

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Nutritional competence is the ability of bacterial cells to utilize exogenous double-stranded DNA molecules as a nutrient source. We previously identified several genes in *Escherichia coli* that are important for this process and proposed a model, based on models of natural competence and transformation in bacteria, where it is assumed that single-stranded DNA (ssDNA) is degraded following entry into the cytoplasm. Since *E. coli* has several exonucleases, we determined whether they play a role in the long-term survival and the catabolism of DNA as a nutrient. We show here that mutants lacking either ExoI, ExoVII, ExoX, or RecJ are viable during all phases of the bacterial life cycle yet cannot compete with wild-type cells during long-term stationary-phase incubation. We also show that nuclease mutants, alone or in combination, are defective in DNA catabolism, with the exception of the ExoX$^-$ single mutant. The ExoX$^-$ mutant consumes double-stranded DNA better than wild-type cells, possibly implying the presence of two pathways in *E. coli* for the processing of ssDNA as it enters the cytoplasm.

Bacteria occupy virtually all niches capable of supporting life, usually in environments where nutrient availability is poor and competition for resources is intense (12). Therefore, starvation is a common state for most bacterial populations for the majority of their existence. Such conditions can be approximated by long-term incubation in the stationary phase of the bacterial life cycle (12).

We showed previously that "nutritional" competence is beneficial for bacterial cells, specifically *Escherichia coli* cells, where extracellular double-stranded DNA molecules (dsDNA) can serve as a nutrient source, and it is necessary for competitive survival (13, 25). A model for DNA uptake and catabolism was proposed in which dsDNA binds to the cell, enters the periplasm, and then is transported across the inner membrane where it is processed into single-stranded DNA (ssDNA) as it enters the cytoplasm (6, 7, 9, 25, 28, 29). In the final step, intracellular exonucleases degrade the ssDNA inside the cytoplasm, where degradation products can serve as the sole source of carbon and energy for the cell (13, 25). *E. coli* contains several nucleases that are able to cleave ssDNA and/or dsDNA molecules. For example, exonuclease I, exonuclease VII, and RecJ digest ssDNA (4, 20, 22), and exonuclease X digests both dsDNA and ssDNA (31). It is possible that one or more of these enzymes participate in the digestion of imported DNA during DNA uptake.

We wanted to determine whether known intracellular ssDNA nucleases play a role in the catabolism of DNA as a nutrient. Exonucleases I, VII, and X and RecJ were chosen because they specifically target ssDNA in the cytoplasm (Table 1). Single and multiple null mutants were constructed since these exonucleases can perform redundant functions in DNA repair and recombination (2, 16, 32). Exonuclease mutants showed the stationary-phase-specific competition-defective (SPCD) phenotype (13, 25) during long-term stationary-phase incubation and are defective in the use of dsDNA as the sole source of carbon and energy, with the exception of the ExoX$^-$ single mutant.

MATERIALS AND METHODS

Bacterial strains and plasmids. All experiments were performed with strains derived from the *E. coli* K-12 strain ZK126 and are listed in Table 2.

Gene disruptions. Each gene of interest was replaced either with a cassette expressing chloramphenicol (Cam^r) or kanamycin (Kan^r) resistance using a Red recombinase-mediated system (8) with the following modifications: template DNA for PCRs was not digested before electroporation, and 15 ml of cells (OD_{600}, ~0.6) per knockout were made electrocompetent immediately prior to electroporation (25). Also, a greater number of transformants was obtained when the Red recombinase was induced for 1 h before preparing cells for electroporation. Table 3 lists the primers used to generate PCR fragments containing the Cam^r or Kan^r cassette flanked by regions of homology for each particular gene.

The PCR products were purified prior to electroporation using a QIAquick PCR purification kit (Qiagen). All mutants were colony purified and tested for the absence of the Red recombinase-expressing plasmid pKD46 by plating for antibiotic sensitivity on agar plates containing ampicillin (150 μg/ml). To verify each mutation, PCRs were performed using one primer specific to a region upstream of the insertion point (called "check" primers [Table 3]) and another primer complementary to the sequence of P1 (P1-C, CGAAGCAGCTCCAGC CTACAC) to amplify a band of specific size. Once confirmed, the drug resistance cassettes flanked by the FLP recombination target sites were removed by introducing the FLP recombinase-expressing plasmid pCP20 as specified by the protocol (8), with the minor modification that colonies were additionally streaked on Luria-Bertani (LB) plates to ensure the complete loss of the pCP20 plasmid. Mutants with more than one exonuclease gene eliminated were constructed by the bacteriophage P1-mediated transduction of the Cam^r or Kan^r allele, followed by removal of the Cam^r or Kan^r cassette by FLP recombination as appropriate. Each mutant strain with multiple gene knockouts was verified by PCR.

Long-term survival assays in LB and defined minimal media. *E. coli* wild-type (ZK126) and mutant strains were separately incubated overnight in 5 ml of LB broth (Difco) or in M63 minimal medium containing 0.2% glucose at 37°C with aeration in a humidified warm room (65% relative humidity). Titers were determined by the periodic sampling of the cultures and serial dilution and plating

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TABLE 1. Characteristics of *E. coli* ssDNA exonucleases

Gene[a]	Protein	Polarity	Target	Location (min)
xonA, *sbcA*	ExoI	3′→5′	ssDNA	44.85
recJ	RecJ	5′→3′	ssDNA	65.41
xseA	ExoVII	3′⇆5′	ssDNA	56.74
exoX, *yobC*	ExoX	3′→5′	ssDNA/dsDNA	41.48

[a] Other commonly used gene names are indicated as appropriate.

on LB plates and are accurate to within threefold (19). The limit of detection was <1,000 CFU/ml.

Batch culture competition assays. The *E. coli* wild-type, ZK1142 (Nal^r) or ZK1143 (Str^r), and mutant strains were separately incubated overnight in 5 ml of LB broth (Difco) at 37°C with aeration. Cultures were then diluted 1:1,000 (vol/vol) in fresh LB broth, either individually or in coculture, by inoculating 5 μl of LB broth with each bacterial strain (13). Subpopulation titers were determined by periodic sampling of the cultures, serial dilution, and plating on medium containing appropriate antibiotics (nalidixic acid at 20 μg/ml, streptomycin at 50 μg/ml, Kan at 50 μg/ml, or Cam at 20 μg/ml) to determine the relative fitness (10, 14). All antibiotics were purchased from Sigma-Aldrich. Experiments were performed in triplicate for each mutant strain. The limit of detection in all experiments was <1,000 CFU/ml.

Preparation of minimal medium supplemented with purified DNA. M63 medium was prepared as described previously (24, 26). Sonicated salmon sperm DNA (Sigma-Aldrich) was prepared as previously described (13, 25), including multiple rounds of organic extraction (phenol, phenol-chloroform, chloroform, and ether) to remove the contaminating proteins. For each "DNA eating" experiment, purified salmon sperm DNA was freshly precipitated with ethanol-acetate and resuspended to a final concentration of 0.1% (wt/vol) in 1-ml M63 cultures. Media were prepared and all experiments were performed in new acid-treated glassware to eliminate the possibility of contamination by other nutrients. Cultures were inoculated at a dilution of ~1:10,000,000 (vol/vol; ~10^2 CFU/ml), and viable cell counts were determined periodically for 48 h by plating on LB plates. Experiments were performed several times for each mutant (n = 2 to 4).

RESULTS

Stationary phase survival in LB and M63 media. Based on the DNA eating model, the prediction was made that one or more exonucleases might be involved in the degradation of ssDNA inside the cytoplasm. *E. coli* has four potent exonucleases targeting ssDNA: ExoI, RecJ, ExoVII, and ExoX. To evaluate the role of these enzymes in the catabolism of internalized ssDNA, isogenic null mutants were constructed using the method of Datsenko and Wanner (8). Due to the fact that there is redundancy in the functions of these nucleases, we also created double, triple, and quadruple mutants to observe any deficiency in the DNA consumption process.

It was previously reported that multiple exonuclease mutants are viable, although quadruple mutants show a reduction in viability after incubation at low temperature in rich medium (2, 32). Here we created new mutant strains in order to (i) maintain the isogenicity of the markers used, (ii) remove, as much as was practical, the possible effects of the presence of multiple antibiotic resistance markers which might have differential effects on fitness in competition experiments, and (iii) create prototrophic mutant strains appropriate for use in the DNA eating assays.

Once the mutants were constructed (Table 2), it was crucial to determine the ability of the null mutants to grow in LB broth and minimal medium supplemented with glucose to eliminate the possibility of gross fitness defects as shown previously (2, 32). Figure 1 shows the monoculture growth patterns of the wild type and the null mutants in different media. No significant defects were observed for any mutant strains, including the quadruple mutant, indicating that the exonucleases are not essential for survival under these conditions during the exponential, stationary, or long-term stationary phases.

TABLE 2. Bacterial strains

Strain	Relevant genotype/phenotype[a]	Reference or source
Strains		
ZK126	W3110 Δ*lacU169 tna-2*	34
ZK1142	ZK126 Nal^r	34
ZK1143	ZK126 Str^r	34
SF2526	ZK126 *xonA*::Cam^r	This work
SF2527	ZK126 *recJ*::Cam^r	This work
SF2528	ZK126 *xseA*::Cam^r	This work
SF2529	ZK126 *exoX*::Cam^r	This work
SF2530	ZK126 *xonA*::Kan^r	This work
SF2531	ZK126 *recJ*::Kan^r	This work
SF2532	ZK126 *xseA*::Kan^r	This work
SF2533	ZK126 *exoX*::Kan^r	This work
SF2534	ZK126 *xonA*::FRT	This work
SF2535	ZK126 *recJ*::FRT	This work
SF2536	ZK126 *xseA*::FRT	This work
SF2537	ZK126 *exoX*::FRT	This work
SF2538	ZK126 *exoX*::FRT *xonA*::FRT	This work
SF2539	ZK126 *xseA*::FRT *xonA*::FRT	This work
SF2540	ZK126 *xseA*::FRT *recJ*::FRT	This work
SF2541	ZK126 *exoX*::FRT *xonA*::FRT *recJ*::FRT	This work
SF2542	ZK126 *exoX*::FRT *xonA*::FRT *xseA*::FRT	This work
SF2543	ZK126 *xseA*::FRT *recJ*::FRT *xonA*::FRT *exoX*::FRT	This work
SF2544	ZK126 *xseA*::FRT *exoX*::FRT *xonA*::FRT *recJ*::Cam^r	This work

[a] FRT refers to an eliminated drug resistance cassette using a helper plasmid encoding the FLP recombinase, leaving a single FLP recombination (FRT) site (see reference 8).

Exonuclease mutants show a SPCD phenotype when coincubated with wild-type cells during the long-term stationary phase. Since the exonuclease mutants show similar patterns of long-term survival when incubated in monoculture, we next assayed for the SPCD phenotype, which reflects a deficit in some activity that is only revealed in competition with otherwise wild-type strains during the long-term stationary phase (14, 25). The mutants exhibit no fitness loss during the exponential phase of growth or upon entry into stationary phase when cocultured with the wild-type parent. However, after several days of coincubation in stationary phase with the wild-type cells, all mutants are outcompeted and most of them are completely lost after 2 weeks of continuous batch coculture (Fig. 2). Such a phenotype is indicative of SPCD and shows that exonucleases are important during long-term survival in stationary phase. While the *exoI* mutant shows a reduction in competitive fitness, unlike the other three single mutant strains that are outcompeted, its final population density stabilizes at 10% of that of the wild type. This suggests that the loss of ExoI function is being compensated for by other proteins, perhaps due to functional redundancy or because the role of ExoI is less important under these conditions.

Ability of exonuclease mutants to utilize dsDNA as a sole source of carbon and energy. To address the ability of exonuclease null mutants to utilize dsDNA, a DNA eating assay was

TABLE 3. Primers used to generate null mutations in different exonuclease genes

Gene	Primer[a]	Sequence
xonA	H1P1	ATGATGAATGACGGTAAGCAACAATCTACCTTTTTGTTTGTGTAGGCTGGAGCTGCTTC
	H2P2	GACAATCTCTTCCGCGTACTGCCAAAGTGCTTTTAACAGCATATGAATATCCTCCTTAG
	CHECK	CCAGGACCCGGCATGAATCTCTG
recJ	H1P1	AAACAACAGATACAACTTCGTCGCCGTGAAGTCGATGAAAGTGTAGGCTGGAGCTGCTTC
	H2P2	TGGCCAGATATTGTCGATGATAATTTGCAGGCTGCGGTTGCATATGAATATCCTCCTTAG
	CHECK	GGGATCACCGTGCGTTATCTTGC
xseA	H1P1	TTACCTTCTCAATCCCCTGCAATTTTTACCGTTAGTCGCCGTGTAGGCTGGAGCTGCTTC
	H2P2	CTTTCTATCCAGCCGTCTTCCAGACGTGTGGTTAGCATTTCATATGAATATCCTCCTTAG
	CHECK	CGTTACGCTCGGTCAGTTCTTTCAC
exoX	H1P1	ATGTTGCGCATTATCGATACAGAAACCTGCGGTTTGCAGGTGTAGGCTGGAGCTGCTTC
	H2P2	AGTATTTTCCAGATAATGTTTCAGTGTTAAACGCAGCTCCATATGAATATCCTCCTTAG
	CHECK	GGATCTACCATCAGAGTCATGGTGC

[a] H1 corresponds to approximately the first 12 codons and H2 corresponds to the last 12 codons of each particular gene. P1 and P2 are underlined and correspond to the regions flanking the antibiotic resistance cassette located on the pKD3 and pKD4 plasmids (8).

performed in which the only source of carbon and energy supplied was highly purified sonicated salmon sperm DNA at a concentration of 0.1% (wt/vol) (13, 25). Wild-type cells and different mutants were inoculated at ~100 CFU/ml into minimal medium supplemented with dsDNA and incubated at 37°C with aeration for 2 days. Cell titers were determined over 48 h, and final growth yields were calculated (Table 4). The wild-type cells showed an increase in cell density of more than 200-fold compared to the mutant strains, which showed little or no growth with one exception, the ExoX$^-$ strain. The DNA eating phenotype of the *exoX* single mutant was unexpectedly high, and the mutant showed a growth yield of ~1,450-fold, which is, on average, seven times greater than that of the wild-type cells.

DISCUSSION

The working model for the use of DNA as a nutrient predicts that when extracellular dsDNA is taken up by cells, only ssDNA is ultimately transported into the cytoplasm, where it is degraded by different single-stranded exonucleases; the second strand may be unwound and degraded in the periplasm. We show here that intracellular exonucleases (ExoI, RecJ, ExoVII, and ExoX) play a significant role in the catabolism of DNA as a nutrient, since all strains deficient in any one of them exhibit the SPCD phenotype during long-term coculture with wild-

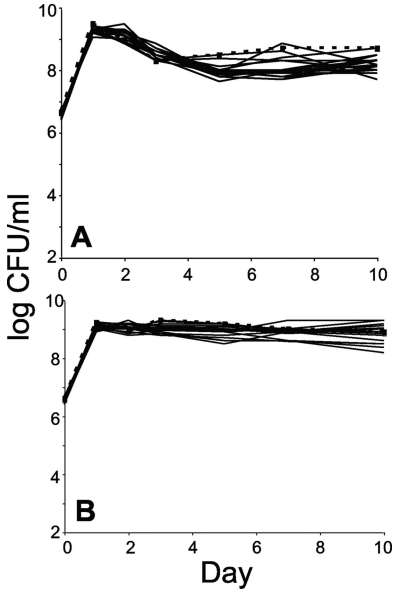

FIG. 1. Exonuclease null mutants show no growth defect during long-term stationary phase incubation. Cells were continuously incubated for 10 days in LB medium (A) or M63 minimal medium supplemented with 0.2% glucose (B). Wild-type cells are represented by filled squares and a dashed line; mutants are represented by solid lines. Each mutant listed in Table 4 is presented here. However, strains are not distinguished since all values agree within the titering error (see reference 19). The limit of detection is <1,000 CFU/ml. Representative data are shown.

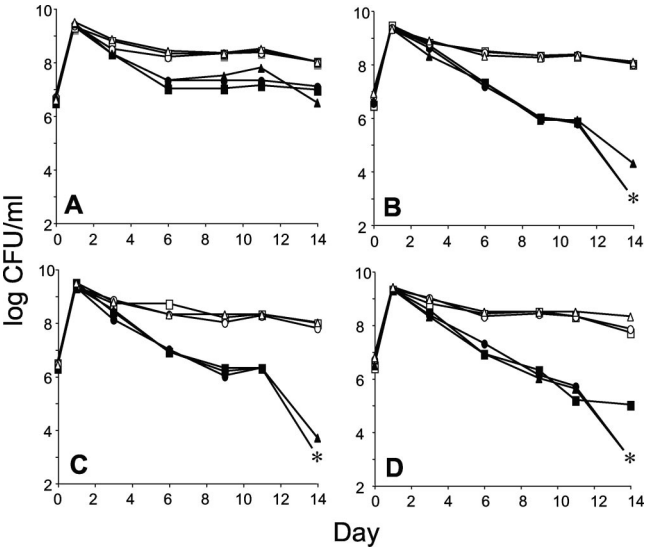

FIG. 2. Survival patterns of different exonuclease mutants competing with wild-type parental strains when cocultured in LB broth. Cultures were continuously incubated for 14 days at 37°C with aeration. Each competition was performed in triplicate; different symbols represent different competition pairs. Strains contain a null mutation for exonuclease I (A), RecJ exonuclease (B), exonuclease VII (C), or exonuclease X (D). Mutant and wild-type strains are represented by filled and open symbols, respectively. Asterisks indicate no detectable counts (the limit of detection is <1,000 CFU/ml).

TABLE 4. Average growth yields of wild-type and exonuclease null mutant cells

Strain	Presence or mutation of[a]:				Average yield (fold) (±SD)[b]
	xonA (ExoI)	*recJ* (RecJ)	*xseA* (ExoVII)	*exoX* (ExoX)	
Wild type	+	+	+	+	223 ± 103
xonA strain	−	+	+	+	3.5 ± 1.1
recJ strain	+	−	+	+	4.0 ± 3.2
xseA strain	+	+	−	+	2.6 ± 0.5
yobC strain	+	+	+	−	1,458 ± 710
xonA yobC strain	−	+	+	−	2.3 ± 1.4
xonA xseA strain	−	+	−	+	2.0 ± 0.2
recJ xseA strain	+	−	−	+	1.4 ± 1.1
xonA recJ yobC strain	−	−	+	−	2.9 ± 0.6
xonA xseA yobC strain	−	+	−	−	2.3 ± 1.4
xonA recJ xseA yobC strain	−	−	−	−	3.0 ± 2.7

[a] +, presence of a gene; −, mutation of a gene. Experiments were performed several times for each mutant ($n = 2$ to 4).

[b] Cells were grown in M63 minimal medium supplemented with 0.1% ultrapure sonicated salmon sperm DNA as the sole source of carbon and energy. Growth yields were determined by dividing the number of cells after 48 h of incubation by the number of cells at inoculation. SD, standard deviation.

type cells. In addition, cells lacking any individual nuclease are altered in their ability to grow in minimal medium containing dsDNA as a carbon and energy source.

Most of these exonucleases are evolutionarily conserved, except for ExoX which is found primarily in the gammaproteobacteria (31; V. Palchevskiy, unpublished observation), and are involved in a variety of cellular functions. ExoI is specific for ssDNA, hydrolyzes DNA through its 3′→5′ activity, and is involved in DNA repair and DNA restriction to control undesirable levels of DNA recombination by suppressing illegitimate recombination (1, 27, 33). RecJ has an essential role in *recBCD*-independent recombination pathways (21). ExoVII is specific for ssDNA and can initiate hydrolysis at either the 3′ or 5′ end of DNA, generating oligonucleotides as products (3–5). In addition, *xseA* mutants have an increased frequency of recombination and are sensitive to nalidixic acid. ExoX is a DNA repair enzyme able to degrade both ssDNA and dsDNA, exhibiting 3′→5′ polarity with high affinity for ssDNA (32). An *exoX* mutant has increased sensitivity to UV irradiation in a background strain lacking several other DNA repair enzymes. ExoX has also been shown to be involved in the stabilization of tandem repeats preventing deletion events (11). All four of these exonucleases are also involved in mismatch-repair where incorrect nucleotides are removed by a strand-specific excision reaction after replication (11, 18, 30).

The DNA eating assay shows that single mutants lacking either ExoI, ExoVII, or RecJ are unable to utilize dsDNA as a sole nutrient source, while *exoX* mutants can consume dsDNA much better than wild-type cells. These findings were unexpected, as it was predicted that due to the redundancy of functions of these exonucleases, a deficiency phenotype for the DNA consumption process might only appear in multiple mutants. The differences observed are not due to altered transcription levels or the presence of extracellular nucleases released into the medium in the different mutant strains (data not shown). Therefore, it is possible that these exonucleases are working as part of a complex, and the removal of any of them disrupts the normal function of a particular complex. For example, perhaps ExoI, ExoVII, and RecJ work together as part of a DNA catabolism system, while ExoX might also play a role in some other process, such as DNA repair, which directs DNA away from the catabolic system. Therefore, when we created the *exoX* mutant, its complex or pathway was disrupted, leaving more DNA available for the catabolic system, resulting in greater growth yields of the *exoX* mutant strain. However, when the *exoX* mutation is combined with any other nuclease mutation, growth yields return to the low values because the catabolic complex is now also disrupted. The interactions of exonucleases with ssDNA-binding protein might be of interest here, since several exonucleases have been shown to interact with ssDNA-binding protein-bound ssDNA (15, 17, 23).

Taken together, the data presented here support a model in which only ssDNA enters the cytoplasm during the DNA eating process. However, apparently, the polarity of degradation appears to be unimportant since the deletion of either 3′→5′ or 5′→3′ exonucleases lacked the ability to catabolize DNA. The fact that the deletion of three of the nucleases studied impairs the cell's ability to "eat" DNA, while the mutation of a fourth nuclease increases DNA catabolism, suggests that these nucleases may function in concert with each other and/or other proteins in heretofore uncharacterized complexes.

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