

A Defect in the Acetyl Coenzyme A↔Acetate Pathway Poisons Recombinational Repair-Deficient Mutants of *Escherichia coli*

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Received 19 June 2004/Accepted 12 November 2004

Recombinational repair-dependent mutants identify ways to avoid chromosomal lesions. Starting with a *recBC(Ts)* strain of *Escherichia coli*, we looked for mutants unable to grow at 42°C in conditions that inactivate the RecBCD(Ts) enzyme. We isolated insertions in *ackA* and *pta*, which comprise a two-gene operon responsible for the acetate↔acetyl coenzyme A interconversion. Using precise deletions of either *ackA* or *pta*, we showed that either mutation makes *E. coli* cells dependent on RecA or RecBCD enzymes at high temperature, suggesting dependence on recombinational repair rather than on the RecBCD-catalyzed linear DNA degradation. Complete inhibition of growth of *pta/ackA rec* mutants was observed only in the presence of nearby growing cells, indicating cross-inhibition. *pta rec* mutants were sensitive to products of the mixed-acid fermentation of pyruvate, yet none of these substances inhibited growth of the double mutants in low-millimolar concentrations. *pta*, but not *ackA*, mutants also depend on late recombinational repair functions RuvABC or RecG. *pta/ackA recF* mutants are viable, suggesting, together with the inviability of *pta/ackA recBC* mutants, that chromosomal lesions due to the *pta/ackA* defect are of the double-strand-break type. We have isolated three insertional suppressors that allow slow growth of *pta recBC(Ts)* cells under nonpermissive conditions; all three are in or near genes with unknown functions. Although they do not form colonies, *ackA rec* and *pta rec* mutants are not killed under the nonpermissive conditions, exemplifying a case of synthetic inhibition rather than synthetic lethality.

The term “synthetic lethals” was introduced by Dobzhansky to describe the relationship between natural alleles in wild-type populations of *Drosophila pseudoobscura* that, having no phenotype individually, are lethal when combined pairwise in the same organism (11). The contemporary analysis of synthetic lethals involves finding secondary mutations inviable in combination with a query mutation (35). Although originally perceived as a way to reveal metabolic redundancies, by identifying the activities that are necessary for survival in the absence of a particular function, analysis of synthetic lethals exposes the contribution of frequently unrelated and mechanistically different metabolic branches towards the same common goal (28, 29). Because of inviability of the double mutant, identification of a synthetically lethal combination facilitates subsequent selection of suppressors, either of inactivation type or multicopy suppressors (3). Identification of suppressors in genes with known functions immediately suggests potential mechanisms of the original synthetic lethality (5, 19). Thus, identification of inviable combinations with a particular mutation is a potentially powerful tool for unraveling the cellular consequences of the corresponding metabolic defect. However, analysis of synthetic lethals, so popular for the yeast *Saccharomyces cerevisiae*, is used relatively infrequently in bacteria (4).

Chromosomal lesions are local DNA lesions with genomic

consequences, because they block chromosomal replication and/or segregation, thus inactivating not only individual genes, in which they happened to occur, but the whole genetic system of the organism (22). Mechanistically, chromosomal lesions comprise DNA lesions that affect both DNA strands of a duplex opposite each other, the so-called two-strand lesions, the best-known examples of which are double-strand DNA breaks and interstrand cross-links (23). To fix chromosomal lesions, all organisms employ recombinational repair (36, 39) as well as some other DNA repair pathways like translesion DNA synthesis (14) and nonhomologous end joining (41). In eubacteria, recombinational repair is the major way to mend chromosomal lesions (23).

In *Escherichia coli*, two separate pathways for the repair of chromosomal lesions exist (23) (Fig. 1). The central step in both pathways is homologous strand exchange between damaged and intact duplexes which is catalyzed by RecA (9). However, the RecA protein itself is incompetent to initiate repair because it cannot recognize chromosomal lesions in their original form. Repair of unfillable single-strand gaps is initiated by the RecFOR complex of activities (34, 43), whereas repair of double-strand breaks is initiated by the RecBCD enzyme (20). Both activities target RecA to the corresponding chromosomal lesions and license RecA to displace single-stranded DNA (ssDNA)-binding protein from ssDNA, which is always associated with chromosomal lesions. The RecA-promoted strand exchange generates a region of hybrid DNA (in which the original chromosomal lesion can be repaired) bracketed by Holliday junctions. Removal of the spent RecA filaments and resolution of Holliday junctions require either RuvABC resolvase or RecG helicase, the two alternative clean-up teams of recombinational repair (23) (Fig. 1).

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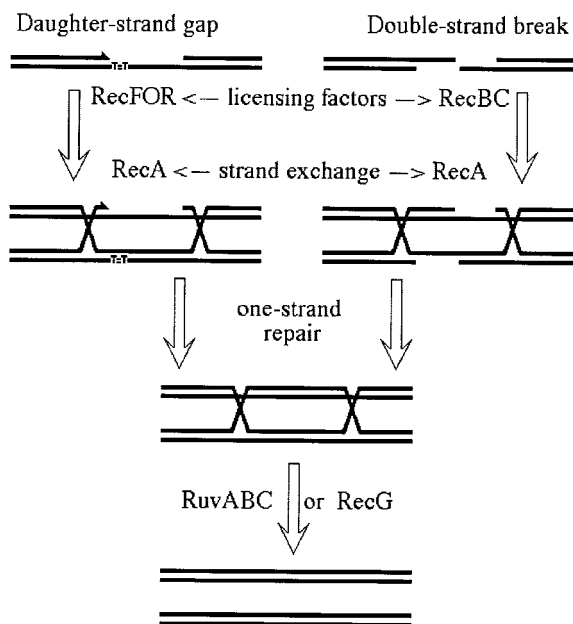


FIG. 1. A scheme of recombinational repair in *E. coli*. RecFOR activities license RecA polymerization of daughter-strand gaps, whereas the RecBC enzyme does the same for double-strand breaks. After the RecA-catalyzed homologous strand exchange enables one-strand repair (excision repair) to fix the irregularities in the individual DNA strands, RuvABC or RecG activities remove the spent RecA filaments and Holliday junctions from the repair intermediate, freeing the participating chromosomes.

Interestingly, although recombinational repair-deficient mutants in *E. coli* cannot mend chromosomal lesions, such mutants are nevertheless viable (7, 33), suggesting the existence of pathways acting to avoid chromosomal lesions. Inactivation of such an avoidance pathway should increase the frequency of chromosomal lesions, making the mutant cells dependent on recombinational repair. Thus, by characterizing *rec*-dependent mutants, it should be possible to identify the metabolic pathways that contribute to the generation of chromosomal lesions as well as the activities that neutralize this “metabolic poisoning” (5, 19, 38). Here we describe the isolation and characterization of two *E. coli* mutants which are dependent on the RecBCD enzyme.

MATERIALS AND METHODS

Media, growth conditions, and general methods. Cells were grown in Luria-Bertani (LB) broth (10 g of tryptone/liter, 5 g yeast extract (YE)/liter, 5 g of NaCl/liter [pH 7.2]) or on LB plates (15 g of agar per liter of LB broth). When necessary, media were supplemented with antibiotics: 50 μ g of kanamycin/ml, 10 μ g of chloramphenicol/ml, or 10 μ g of tetracycline/ml. To determine viability, rapidly growing cultures were serially diluted 10-fold at each step in 1% NaCl, and 10 μ l of every dilution was spotted onto plates and developed under permissive as well as nonpermissive conditions. To detect the effect of cell density, if any, we routinely spotted all dilutions. DNA was kept in TE buffer (10 mM Tris HCl [pH 8.0], 1 mM EDTA).

Bacterial strains. Bacterial strains were *E. coli* K-12 derivatives (Table 1). *pta* and *ackA* deletion-replacement alleles were confirmed by PCR. *recA*(Ts), *recBC*(Ts), Δ *recA*, Δ *recBCD*, Δ *recF*, *recG*, and Δ *ruvABC* mutations were confirmed by their characteristic UV sensitivities. Gene replacements with either chloramphenicol or kanamycin resistance markers were constructed according to the method described by Datsenko and Wanner (10). The sequences of the

primers used in the construction of the replacement alleles are shown in Table 2.

Quantification of CFU. A fresh culture grown overnight at 28°C was serially diluted 10-fold up to 10^{-5} by using 1% NaCl. Five microliters of each dilution was spotted onto an LB plate in the presence of cross-inhibiting streaks of AB1157. After incubating the plate at 45°C for 16 to 24 h, individual spots at a 10^{-1} dilution were punched out, shaken at 28°C in 1 ml of 1% NaCl for 1 h to disperse the cells, serially diluted, spotted onto LB plates, and grown at 28°C for 16 to 24 h. Individual colonies were then counted to determine the number of CFU in the original spot grown at 45°C. The values were not normalized to the titer of the original culture that was grown overnight since we found that the titers of the cultures grown overnight were almost the same ($\pm 10\%$), independent of the genotype of the strain.

Pulsed-field gel electrophoresis. Cultures of strains to be tested, which were grown at either 28 or at 45°C, were normalized to an optical density at 600 nm (OD_{600}) of 0.3. Cells from 1.5 ml of the normalized cultures were sedimented by centrifugation and resuspended in 60 μ l of TE buffer. After incubation at 37°C for 2 min, 5 μ l of 10 mg of proteinase K/ml was added, followed by 65 μ l of 1.2% molten agarose in 0.2% sarcosyl–10 mM Tris HCl (pH 8.0)–5 mM EDTA (kept at 70°C). After vortexing and mixing by pipetting, 110 μ l of the cell suspension in agarose was pipetted into a plug mold and was allowed to solidify for 2 to 10 min. Each plug was then placed in a small glass tube containing 1 ml of 1% sarcosyl–50 mM Tris-HCl (pH 8.0)–25 mM EDTA and incubated for 1 h at 60°C. Plugs were inserted into 1% agarose gel on 0.5 \times Tris-borate-EDTA buffer and run in a Gene Navigator unit (Amersham-Pharmacia) with hexagonal electrodes (10°C, 180 V, ramp 3 to 165 s, 20 h).

Insertional mutagenesis. For Tn5 mutagenesis, 10^9 cells of SK129 [AB1157 *recBC*(Ts)] carrying λ cI-expressing plasmid pKB252 (2) were infected with 10^8 particles of Tn5-carrying λ (MMS704 [Δ b221 *rex*::Tn5 *cI857*], from Frank Stahl’s collection) in a total volume of 150 μ l of TM buffer (10 mM Tris HCl [pH 8.0], 10 mM $MgSO_4$), giving a multiplicity of infection of 0.1. After 15 min on ice, injection was effected at 37°C for 5 min, and the adsorption mixture was transferred to 1.5 ml of LB broth and shaken at 28°C for 30 min. The culture was plated onto kanamycin- and tetracycline-supplemented LB plates which were incubated at 28°C for 2 days, and the colonies were screened for the inability to grow at 42°C on LB plates by toothpicking. The kanamycin resistance determinant from the temperature-sensitive candidates was P1 transduced into the *recBC*(Ts) parent to confirm the associated temperature sensitivity, indicative of dependence on the RecBCD enzyme. The position of the insert was identified by sequencing out of the transposon (using primer 93seq [5’-GCCAGTCGGCC GCACGATG]) after subcloning the transposon halves with the neighboring chromosomal sequences and selection for the associated kanamycin resistance.

For an alternative insertional mutagenesis, we used pRL27, a plasmid carrying a hyperactive Tn5 transposase gene adjacent to the insertional cassette comprising two inverted Tn5 termini bracketing the *kan* gene plus the R6K *pir*-dependent origin (25). The cells of the strain to be mutagenized were electroporated with 10 ng of pRL27 and plated for kanamycin resistance with an immediate selection for the desired phenotype. Once the clones of interest were identified, total DNA from 2 ml of cultures grown overnight were prepared as described previously (24), and 1/20 of it was digested with MluI, ligated onto itself, and electroporated into DH5 α *pir*⁺ cells with selection for kanamycin resistance. The retrieved plasmids were digested with MluI, and the smallest plasmid was selected for sequencing. The two primers used for sequencing of pRL27 inserts were 27 ori (5’-AACAAAGCCAGGGATGTAACG [the *ori* side of the insert]) and 27 kan (5’-CAGCAACACCTTCTTACGA [the *kan* side of the insert]).

Enrichment for mutants inhibited in the absence of RecBC. The enrichment for mutants inhibited in the absence of RecBC is based on the observation that recombination-dependent mutants, although unable to form colonies when recombinational repair is inactivated, maintain viability for several hours and resume growth once the recombinational repair capacity is restored. Transient growth inhibition due to recombinational repair deficiency allows recombination-dependent mutants to survive treatments with antibiotics, like ampicillin, that kill growing cells. To enrich for *recBC*-dependent mutants, *recBC*(Ts) cells were mutagenized (as described above) and plated onto LB agar supplemented with kanamycin to obtain $\sim 1,000$ colonies per plate. The next day, 1 ml of LB broth per plate was used to wash off and combine $\sim 5,000$ colonies. The cell suspension was diluted to 10^7 cells/ml into 40 ml of LB broth, and the culture was pregrown at 42°C for 1 h to inhibit *rec*-dependent cells. At this point, ampicillin was added to 100 μ g/ml, and incubation at 42°C was continued for 4 more hours, at which point the culture cleared, indicating cell lysis. The surviving cells were collected by centrifugation (15,000 \times g, 10 min) and resuspended in 1 ml of LB broth without ampicillin. Fifty microliters of various dilutions of this suspension was spread to single colonies on LB plates supplemented with kanamycin and

TABLE 1. Strains

Strain	Background ^a	DNA metabolism	Source, derivation, or reference
Previous studies			
AB1157	AB1157	Rec ⁺	1
AK4	AB1157	$\Delta(\text{srlR-recA})306::\text{Tn10}$	This laboratory
AM3	AB1157	$\Delta\text{recF20}::\text{cat}$	33
AM547	AB1157	$\Delta(\text{ruvA-C})65$	26
DH5 $\alpha::\text{pir}$		<i>pir</i> ⁺	32
JB1	AB1157	$\Delta\text{recBCD3}::\text{kan}$	33
JC9239	AB1157	<i>recF143</i>	17
JC9941	AB1157	<i>recA200</i> (Ts)	A. J. Clark
L-56	AB1157	<i>recA200</i> (Ts) $\Delta\text{mltB1085}::\text{cat}$	19
N2057	AB1157	<i>ruvA60}::\text{Tn10}</i>	26
N4452	AB1157	$\Delta\text{recG265}::\text{cam}$	31
SK129	AB1157	<i>recB270</i> (Ts) <i>recC271</i> (Ts)	21
TNM1072	AB1157	<i>recG263}::\text{kan}</i>	26
This study			
JB17	AB1157	$\Delta\text{pta51}::\text{cat}$	Deletion-replacement (pKD46)
JS1	AB1157	$\Delta\text{pta51}::\text{cat recB270$ (Ts) <i>recC271</i> (Ts)	SK129 \times P1 JB17
JS2	AB1157	$\Delta\text{pta51}::\text{cat recA200$ (Ts)	JC9941 \times P1 JB17
JS4	AB1157	$\Delta\text{pta52}::\text{kan}$	Deletion-replacement
JS5	AB1157	$\Delta\text{pta52}::\text{kan recB270$ (Ts) <i>recC271</i> (Ts)	SK129 \times P1 JS4
JS6	AB1157	$\Delta\text{pta52}::\text{kan recA200$ (Ts)	JC994 \times P1 JS4
JS7	AB1157	$\Delta\text{pta52}::\text{kan } \Delta(\text{ruvA-C})65$	AM547 \times P1 JS4
JS8	AB1157	$\Delta\text{pta52}::\text{kan recF143}$	JC9239 \times P1 JS4
JS9	AB1157	$\Delta\text{pta52}::\text{kan } \Delta\text{recG265}::\text{cat}$	N4452 \times P1 JS4
JS12	AB1157	$\Delta\text{pta52}::\text{kan } \Delta(\text{srlR-recA})306::\text{Tn10}$	JS4 \times P1 AK4 (pEAK2)
JS13	AB1157	$\Delta\text{pta51}::\text{cat } \Delta\text{recBCD3}::\text{kan}$	JB17 \times P1 JB1 (pK134)
IYS1	AB1157	$\Delta\text{pta51}::\text{cat recBC$ (Ts)	SK129 \times P1 JB17
IYS2	AB1157	$\Delta\text{pta51}::\text{cat recA200$ (Ts)	JC9941 \times P1 JB17
IYS3	AB1157	$\Delta\text{pta52}::\text{kan}$	AB1157 \times P1 JS4
IYS4	AB1157	$\Delta\text{ackA45}::\text{kan}$	Deletion-replacement (pKD46)
IYS5	AB1157	<i>ackA46</i>	IYS4 <i>kan</i> removal (pCP20)
IYS6	AB1157	$\Delta\text{ackA45}::\text{kan recBC$ (Ts)	SK129 \times P1 IYS4
IYS7	AB1157	$\Delta\text{ackA45}::\text{kan recA200$ (Ts)	JC9941 \times P1 IYS4
IYS9	AB1157	$\Delta\text{ackA45}::\text{kan } \Delta\text{recA304}$	JC10287 (pBEU14) \times P1 IYS4
IYS11	AB1157	$\Delta\text{ackA45}::\text{kan } \Delta\text{recF20}::\text{cat}$	AM3 \times P1 IYS4
IYS14	AB1157	$\Delta\text{ackA45}::\text{kan ruvA60}::\text{Tn10}$	IYS4 \times P1 N2057
IYS15	AB1157	$\Delta\text{ackA46 recG263}::\text{kan}$	IYS5 \times P1 TNM1072

^a Complete genotypes of backgrounds are as follows: AB1157, F⁻ λ ⁻ *rac*⁻ *thi-1 hisG4* $\Delta(\text{gpt-proA})62$ *argE3 thr-1 leuB6 kdgK51 rfbD1 araC14 lacY1 galK2 xylA5 mtl-1 tsx-33 supE44(glnV44) rpsL31 (strR)*; DH5 $\alpha::\text{pir}$, F⁺ $\lambda::\text{pir endA1 hsdR17}(r^- m^+)$ *glnV44 thi-1 recA1 relA1* $\Delta(\text{lacIZYA-argF})$ U169 *deoR*(ϕ 80- $\Delta\text{lacZM15}$) *gyrA*(Na1R).

incubated at 28°C. The next day, 100 colonies were toothpicked to check for the inability to grow at 42°C. One of the multiple temperature-sensitive clones thus obtained (that were assumed to be all siblings) was saved for further analysis.

Sandwich plating. A 25-ml LB plate was overlaid with a 3.5-ml layer of top agar containing 300 μ l of a mid-log-phase AB1157 culture ($\sim 1 \times 10^8$ cell/ml) and incubated at 37°C for 4 h. The plate was then overlaid with another 3.5 ml of top agar containing 175 μ l of 100 \times stock of kanamycin (to eventually produce 25 μ g of kanamycin/ml per entire ~ 36 -ml plate). After 2 min, a third 3.5-ml layer of top agar containing 500 μ l of the pRL27-electroporated culture of the *pta recBC*(Ts) mutant was added. The plate was then incubated at 42°C for 36 to 48 h.

RESULTS

Isolation of mutants inhibited in the absence of RecBCD enzyme. The *recBC*(Ts) mutants can grow at 42°C as long as they do not suffer chromosomal lesions that require recombinational repair. To find mutants that suffer from chromosomal lesions and that are therefore dependent on recombinational repair, random Tn5 insertions were selected in a *recBC*(Ts) strain at 28°C (permissive temperature) and screened for the

TABLE 2. Sequences of primers used for *ackA* and *pta* gene replacement

Purpose	Name	Sequence ^a
<i>pta</i> deletion	pta-for	CGAGCCGCTGACTGCCTGATTTACACCCGCGCAGCTCAGCGTGTAGGCTGGAGCTGCCTC
<i>pta</i> deletion	pta-rev	GTATGCAAAGTGGGATGGCGCAATTCATTGATGCAGCGCAGCATATGAATATCCTCCTTAG
<i>pta</i> verification	ptaF2	CTGGCTGCACGTTTCGGC
<i>pta</i> verification	ptaR2	GTTGATTGCGGACATAGCGC
<i>ackA</i> deletion	ACK1	CTGACGTTTTTTTAGCCACCGTATCAATTATAGGTACTTCCCTGTAGGCTGGAGCTGCCTTCG
<i>ackA</i> deletion	ACK2	TGATGAAACCAGATTTGCCGAAACGTGCAGCCAGGTTGCCATATGAATATCCTCCTTAG
<i>ackA</i> verification	VP-ack-1	CATGCGCTACGCTCTATGGC
<i>ackA</i> verification	VP-ack-2	GGCAGTCAGGCGGCTCGC

^a The nucleotides complementary to the pKD3/4 templates are underlined.

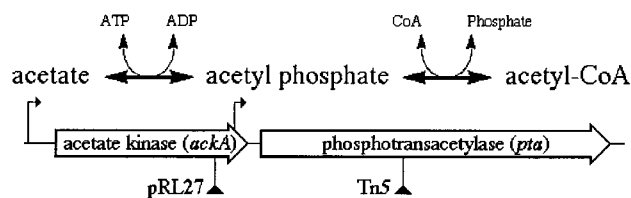


FIG. 2. A scheme of acetate↔acetyl-CoA conversion and the genes involved. The elements of the *ackA-pta* operon are drawn to scale. The *ackA* gene is 1,202 bp, whereas the *pta* gene is 2,145 bp. The two promoters are shown as small arrows on long vertical stems. The positions of pRL27 and Tn5 inserts are indicated by filled triangles on vertical stems.

inability to grow at 42°C (nonpermissive temperature), yielding an insertion in *pta* (Fig. 2). In an enrichment procedure for mutants unable to grow in combination with *recBC* inactivation at 42°C, a *kan* (pRL27) insertion into *ackA* was isolated (Fig. 2). Since the *ackA* and *pta* genes form a two-gene operon in *E. coli*, our isolation of the two mutations as inhibited at 42°C in the absence of RecBCD enzyme activity suggested that the *ackA-pta* pathway is somehow involved in the avoidance of chromosomal lesions in *E. coli*.

The *ackA* gene encodes acetate kinase, an enzyme that catalyses the interconversion of acetate and acetyl-phosphate. The downstream *pta* gene codes for phosphotransacetylase, an enzyme that catalyzes the interconversion of acetyl-phosphate and acetyl coenzyme A (acetyl-CoA). In effect, the *pta-ackA* pathway converts acetyl-CoA via acetyl-phosphate into acetate or vice versa, with the direction of the overall reaction dictated by growth conditions (Fig. 2) (6, 12, 37). In a minimal medium with acetate as the sole energy source, the *ackA-pta* pathway charges acetate on CoA, thus enabling acetate to enter the metabolism. In a rich medium, the pathway reverses (acetyl-CoA is converted to acetate), allowing the cell to dump the excess of acetylation potential in exchange for energy in the form of ATP. We set out to characterize the *rec* dependence of *pta* and *ackA* mutants in *E. coli* and aimed to identify the type of chromosomal damage involved when the *ackA-pta* pathway is inactivated.

Chromosomal fragmentation in *ackA*⁺ and *ackA* mutant cells. Deletion-replacement alleles $\Delta ackA::kan$ and $\Delta pta::kan$ with the corresponding open reading frames precisely removed as well as an $\Delta ackA$ allele with the drug resistance gene removed were constructed. Both $\Delta ackA$ (data not shown) and Δpta (Fig. 3A) mutations made the original *recBC*(Ts) strain unable to grow at 42°C, confirming the observations made with Tn5 and pRL27 insertions.

In terms of the chromosome, dependence of a mutant on the RecBCD enzyme indicates the formation of double-strand DNA breaks, which translates into chromosomal fragmentation, detectable by pulsed-field gel electrophoresis (5, 19, 30). In pulsed-field gels, intact chromosomal DNA stays in the wells, whereas subchromosomal fragments migrate into the gel, forming a characteristic smear. We carried out pulsed-field gel electrophoresis of DNA isolated from *ackA recBC*(Ts) double mutants grown at 45°C as well as from the corresponding single mutants and wild-type cells. There was definitely more chromosomal fragmentation in the *ackA recBC*(Ts) double mutant than in the *ackA* single mutant (Fig. 3B, compare lanes

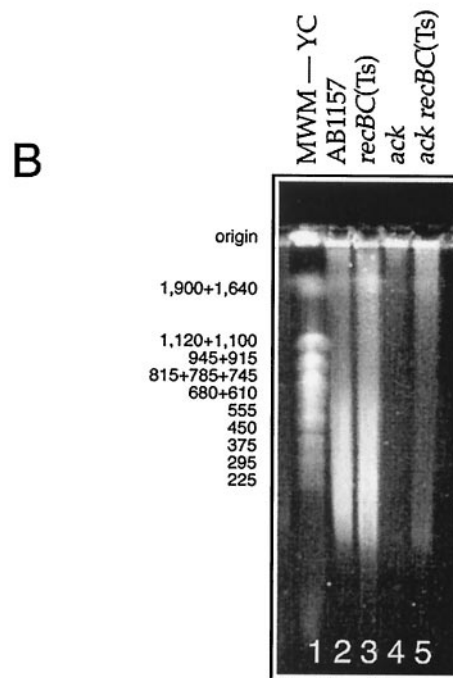
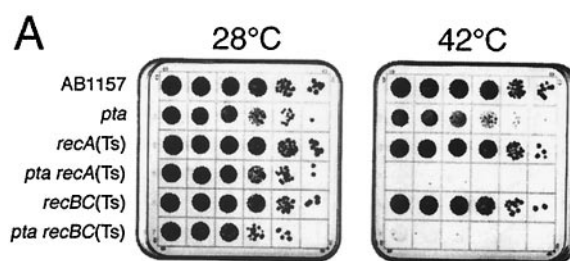


FIG. 3. $\Delta pta/\Delta ackA$ *recBCD* mutants are inviable and fragment their chromosome. A. *pta recA*(Ts) and *pta recBC*(Ts) are inviable at 42°C. Serial 10-fold dilutions of growing cultures were spotted onto plates which were incubated at the indicated temperatures for 36 to 48 h. The strains are as follows: for *pta*, JB17; for *recA*(Ts), JC9941; for *pta recA*(Ts), JS2; for *recBC*(Ts), SK129; for *pta recBC*(Ts), JS1. B. Pulsed-field gel of chromosomal DNA isolated from AB1157, *recBC*(Ts), $\Delta ackA$, and $\Delta ackA recBC$ (Ts). The cells were grown at 45°C to an OD₆₀₀ of 0.3. MWM—YC, yeast chromosomes serving as molecular weight markers (their length in kilobase pairs is indicated on the left). The strains are as follows: for *recBC*(Ts), SK129; for *ackA*, IYS4; for *ackA recBC*(Ts), IYS6.

4 and 5). However, we have encountered two types of difficulties with quantification of chromosomal fragmentation in *ackA recBC*(Ts) double mutants: (i) an unexpectedly high level of chromosomal fragmentation in wild-type and *recBC*(Ts) mutant cells at 45°C (the temperature we had to use to obtain the *ackA* mutant phenotype [see below]) (Fig. 3B, lanes 2 and 3) and (ii) a much reduced chromosomal fragmentation in *ackA* mutants compared to that in the *ackA*⁺ cells, most likely due to the slow growth of the mutant (Fig. 3B, compare lanes 2 and 4 and lanes 3 and 5). These results suggest that (i) there is chromosomal instability of unknown origin in the wild-type cells at 45°C and (ii) the apparent inhibition of chromosomal fragmentation in *recBC*(Ts) mutant cells by the *ackA* mutation

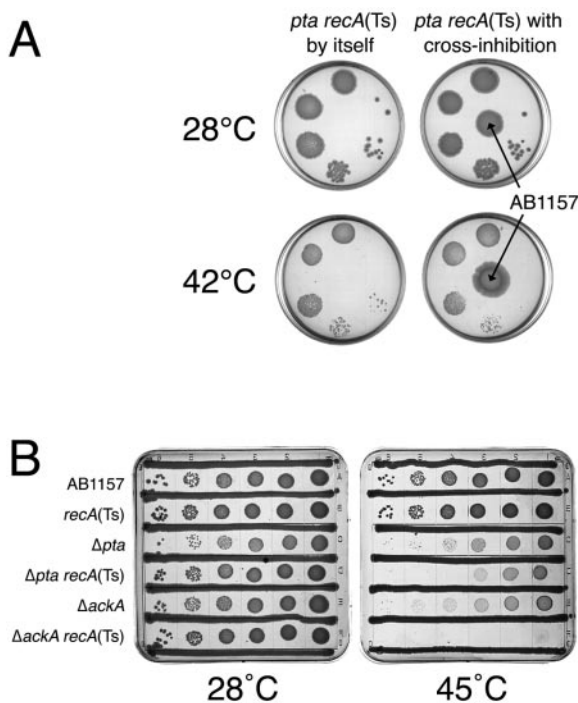


FIG. 4. *pta/ackA rec* mutants are inviable at temperatures of 42°C and higher in the presence of growing cells. Serial 10-fold dilutions of growing cultures were spotted onto plates which were incubated at the indicated temperatures. Where present, cross-inhibiting spots or streaks were those of AB1157. A. *pta recA(Ts)* is viable at 42°C when spotted onto a plate without other strains present. Incubation at both temperatures was done for 36 h. Note cross-inhibition from the AB1157 spot (the bottom-right plate). The strain is JS2. B. A typical cross-inhibition plate. Incubation was done for 36 h at 28°C but for only 24 h at 45°C. The strains are as follows: for *recA(Ts)*, JC9941; for Δ *pta*, JS4; for Δ *pta recA(Ts)*, JS6; for Δ *ackA*, IYS4; for Δ *ackA recA(Ts)*, IYS7.

is due to the slow rate of DNA replication in *ackA* mutants. The fragmentation of the chromosome in wild-type cells at 45°C was particularly unexpected and warrants further investigation; however, such an investigation is beyond the scope of this project.

***ΔackA* and *Δpta* mutants depend on RecA and RecBCD.** In terms of DNA repair, dependence of an *E. coli* mutant on RecBCD enzyme indicates that the mutant relies on either linear DNA degradation or recombinational repair of DNA nicks or breaks. If the former is true, the mutant is viable in combination with *recA* inactivation (18, 40); if the latter is the case, the mutant requires RecA as well as RecBCD (5, 16, 19). To see whether *ackA* and *pta* mutants depend on DNA degradation capabilities or recombinational repair capabilities of RecBCD, we combined them with the *recA(Ts)* mutation and found that the resulting strains are inviable at 42°C (Fig. 3A and data not shown), suggesting the dependence of *ackA-pta* mutants on recombinational repair.

***pta* and *ackA* mutants are sensitive to cross-inhibition.** Remarkably, when we spot tested *ackA rec* (data not shown) or *pta rec* (Fig. 4A) cells at 42°C or even at 45°C on individual LB plates rather than on a common plate with other strains, the double mutants were able to form small colonies, suggesting

that the inability of the same strains to form colonies on the original plates was due to cross-inhibition from the growing neighbors (which can be seen on the lower-right plate shown in Fig. 4A). To eliminate possible inconsistencies due to this cross-inhibition by neighbors, from then on, we scored *ackA rec* and *pta rec* double-mutant phenotypes on plates with cross-inhibiting streaks of AB1157, which made the mutant phenotype unequivocal (Fig. 4B).

***pta/ackA rec* mutants are sensitive to medium modification.** The cross-inhibition of *pta/ackA rec* mutants could be due to either exhaustion of critical medium components or, alternatively, excretion of inhibitory waste substances by nearby growing cells. When we grew various strains on “spent” LB agar (AB1157 was grown to saturation, the cells were removed, and the medium was made solid by the addition of agar), all strains grew (slowly) at 28°C; at 42°C, the *pta* single mutant grew extremely poorly, while *pta rec* double mutants failed to grow completely (Fig. 5A, rows 13 to 18). When we grew the same strains on partially spent (“mid-log” LB) medium (obtained by growing AB1157 in LB medium until an OD₆₀₀ of 0.5 was reached, removing cells, and making a solid medium out of such a “preconditioned” LB medium), the effect was opposite: at 42°C, the double mutants grew almost as well as single mutants and actually grew better than they did on the regular LB agar with cross-inhibition (Fig. 5A, compare rows 7 to 12 with rows 1 to 6), as if *pta* mutants were helped by the products excreted by mid-log-phase cells.

We also found that of the two major components of LB agar, tryptone enhanced the growth of the double mutants (data not shown), while YE inhibited it. This inhibition was so strong that on the medium composed of only 0.5× YE (without tryptone), a *pta recA(Ts)* mutant would not grow at 42°C even in the absence of cross-inhibition (Fig. 5B, lower-left plate). At the same time, all other strains tested, including single *pta* mutants, had no problem growing on this yeast extract medium (data not shown). We conclude that *ackA rec* and *pta rec* mutants at 42°C are sensitive to products accumulated in stationary-phase cultures and some components of the yeast extract. Alternatively, the double mutants may require some substances in tryptone that are exhausted by other cells growing nearby.

***pta rec* mutants are sensitive to products of mixed-acid fermentation of pyruvate.** When grown aerobically in rich medium, *E. coli* turns pyruvate exclusively into acetyl-CoA and then uses the *pta-ackA* pathway to dump the excess acetate, which is excreted and then reused as the cells enter stationary phase (8, 12). To test the idea that cross-inhibition is due to *pta rec* double mutant sensitivity to acetate excreted by their *pta*⁺/*ackA*⁺ neighbors, we plated *pta recA(Ts)* and *pta* single-mutant strains at 42°C on LB plates supplemented with increasing concentrations of acetate. We found that the double mutant could not grow in the presence of acetate in excess of 50 to 60 mM, whereas the single mutants grew normally in the presence of 150 mM acetate (Table 3). Still, the inhibiting concentration of acetate was at least 10 times higher than the maximal concentration of acetate that accumulated in cultures of rapidly growing cells (8, 45). Moreover, the *pta/ackA rec* double-mutant strains were inhibited equally well by growing *pta/ackA* single-mutant neighbors (data not shown), ruling out acetate as the inhibitor.

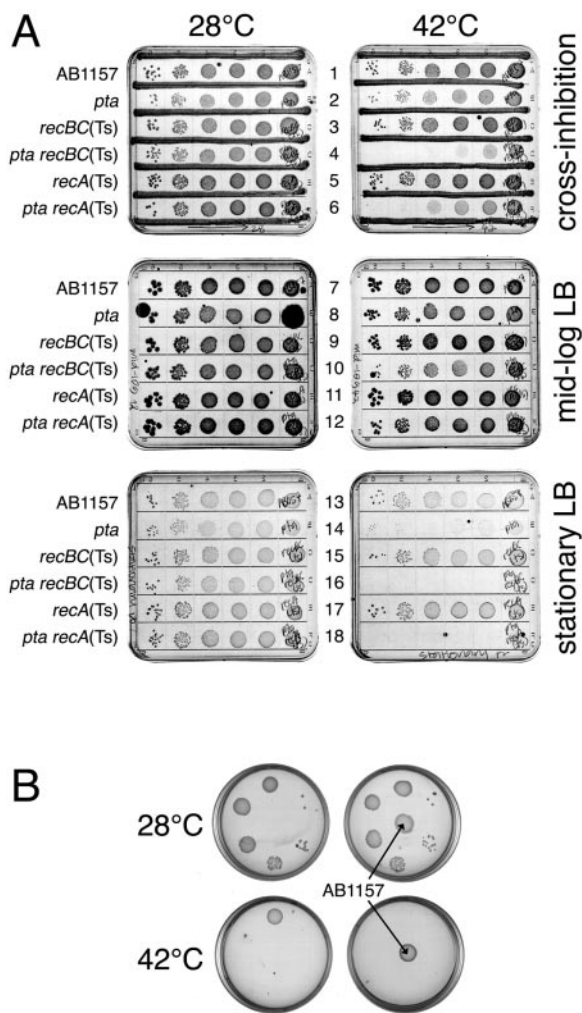


FIG. 5. *pta recA*(Ts) and *pta recBC*(Ts) cells cannot grow at 42°C on spent medium or on medium containing only yeast extract. Serial 10-fold dilutions of growing cultures were spotted onto plates which were incubated at the indicated temperatures for 36 to 48 h. Where present, cross-inhibiting spots or streaks were those of AB1157. A. *pta* mutants grow better on a mid-log medium but cannot grow on a spent medium. Stationary LB, a sterilized supernatant of a saturated AB1157 culture solidified with 1.5% agar; mid-log LB, a sterilized supernatant of a growing AB1157 culture (OD₆₀₀ of 0.5) solidified with 1.5% agar. The strains are as follows: for *pta*, IYS3; for *recBC*(Ts), SK129; for *pta recBC*(Ts), IYS1; for *recA*(Ts), JC9941; for *pta recA*(Ts), IYS2. B. Yeast extract medium does not support growth of a *pta recA*(Ts) mutant at 42°C. The medium contains (per liter) 2.5 g of YE, 5 g of NaCl, and 15 g of agar (pH 7.2). The strain is JS6.

In an attempt to find the substance excreted by the cells growing on LB plates which would inhibit *pta rec* double mutants in low concentrations, we considered a possibility that cells growing in colonies experience a microaerobic environment, which we thought might lead to the activation of mixed-acid fermentation of pyruvate, resulting in excretion of not only acetate but also lactate, ethanol, formate, succinate, and pyruvate itself (Fig. 6) (45). We found that wild-type cells as well as single *pta* and *recA*(Ts) mutants can grow on LB plates supplemented with high concentrations of these various metabolites (Table 3). In contrast, *pta recA*(Ts) mutants are com-

TABLE 3. Concentrations of various metabolites that completely inhibit growth of *pta recA*(Ts) mutants at 42°C

Metabolite	Concn that inhibits growth completely (mM) ^a	
	<i>pta recA</i> (Ts)	<i>pta</i> ^b
(Sodium) acetate	50	>150
(Ammonium) acetate	60	>150
Ammonium chloride	60	>150
Ethanol	3.0%	>5.0%
Lactic acid	0.05%	>0.1%
(Sodium) formate	85	120
(Sodium) pyruvate	60	>150
(Sodium) succinate	80	>150

^a Total concentration per plate in mM or in percent (where indicated). Concentrations that still fail to inhibit *pta* single mutants are shown for comparison.
^b Wild-type cells and *recA*(Ts) mutants were also not inhibited at these concentrations (in excess of 150 mM for salts, 5.0% for ethanol, and 0.1% for lactic acid).

pletely inhibited by lower concentrations of all these metabolites, suggesting that cross-inhibition could indeed be due to excretion of these products into the medium surrounding growing cells. However, there was not a single metabolite among those we tested that would inhibit the double *pta recA*(Ts) mutant in submillimolar or even low-millimolar concentrations (expected during cross-inhibition). Moreover, we found that even ammonium chloride inhibits *pta recA*(Ts) mutants in similar concentrations (Table 3), suggesting that these mutants may generally be sensitive to salts in concentrations that are not inhibitory to *pta* or *rec* single mutants.

***ackA rec* and *pta rec* double mutants are synthetically inhibited rather than synthetically lethal.** The inability to form colonies under nonpermissive conditions (inviability) may represent either death of the cells (lethality) or inhibition without actual loss of viability. The two alternatives can be distinguished by first subjecting cells to nonpermissive conditions, making sure they do not form colonies, and then shifting the cells to permissive conditions to detect decreases in colony-forming ability. Tests like these, conducted previously with *dut-1 rec* and *tdk rec* mutants (E. A. Kouzminova and A. K.

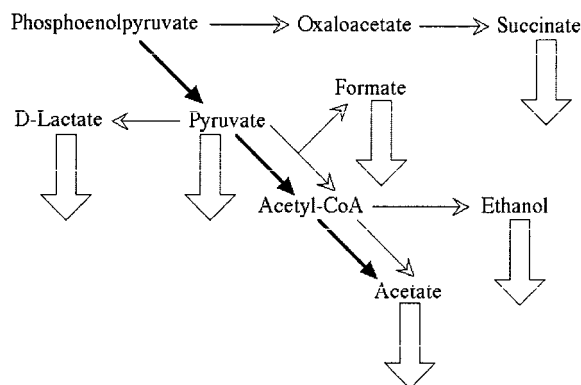


FIG. 6. Metabolic neighbors of the *pta-ackA* pathway during anaerobic growth in a rich medium. The aerobic overflow pathway (phosphoenolpyruvate→pyruvate→acetyl-CoA→acetate) is indicated by boldface arrows. The anaerobic mixed-acid fermentation is shown by thin arrows with open arrowheads. Excreted products are marked by thick downward open arrows. Only the key substances are shown.

Kuzminov, unpublished data), demonstrated quantitative killing of the double mutants under the nonpermissive conditions. However, when we returned *pta rec* or *ackA rec* mutants to permissive conditions, not only did they form colonies, but also the titer of the CFU was the same as that in cultures incubated throughout under permissive conditions (data not shown). Thus, *pta rec* and *ackA rec* mutants showed “synthetic inhibition” rather than synthetic lethality.

To quantify this inhibition, we spotted serial dilutions of saturated cultures, grew the spots under inhibitory conditions, and determined the titer of the CFU in the “-2” dilution spot at the permissive conditions (Fig. 7A). The titers of single *rec* or *ackA* mutant spots determined by this procedure were similar to the titer of spots of wild-type cells (Fig. 7B). However, the titer of *ackA rec* mutant spots was down by 2 orders of magnitude. Thus, although *ackA rec* and *pta rec* mutant cells do not lose viability in the nonpermissive conditions, they in effect form 100-times-smaller colonies, which makes them almost invisible. We suggest that *ackA rec* and *pta rec* combinations be called synthetically inhibited rather than synthetically lethal.

***pta* mutants depend on RecG and RuvABC but not RecF.** As pointed out above, recombinational repair of chromosomal lesions has three phases (Fig. 1). First, the targeting or licensing activities of RecBCD and RecFOR recognize specific chromosomal lesions and target the RecA protein to these lesions, licensing it to displace ssDNA-binding protein from the lesion-associated ssDNA. Second, RecA filament catalyzes homologous pairing and strand exchange between the affected and the intact DNA duplexes, forming hybrid duplexes between Holliday junctions. Third, after the now one-strand DNA lesions have been repaired in the hybrid duplexes, RuvABC or RecG activities remove spent RecA filaments and resolve the DNA junctions, restoring functional chromosomes. Our demonstration of the RecA and RecBC dependence of *pta* and *ackA* mutants prompted us to see if other recombinational repair activities are required when the acetyl-CoA \leftrightarrow acetate pathway is inactivated. We found that *ackA* and *pta* mutants do not require RecF, either with or without cross-inhibition (Fig. 8 and data not shown), suggesting that the chromosomal lesions in these mutants do not include daughter-strand gaps. On the other hand, we found that *pta* mutants depend on both RecG and RuvABC (with cross-inhibition at 42°C) (Fig. 8). This result is unexpected because the late phase of recombinational repair is believed to be catalyzed by either activity, so RecA-dependent mutants usually show dependence on both late activities but could survive if one of them is still active. In contrast to the Δ *pta* mutant, our Δ *ackA* mutant was viable in combination with either *recG* or *ruvABC* mutations (data not shown), which was the only difference in phenotypes between *pta* and *ackA* mutants detected in this work.

Curiously, although the *recG* and *ruv* mutations we used were complete inactivations and the double mutants with *pta* were inviable at 42°C, they grew normally at 37°C (Fig. 8), suggesting a temperature-sensitive nature of the *pta* (and, by analogy, *ackA*) defect. To verify this suspicion, we attempted to build Δ *recA pta/ackA* and Δ *recBCD pta* strains at low temperature, and we indeed recovered the double mutants. The mutants were fully viable at 28 or even 37°C but, as expected, were unable to form colonies at 42 or 45°C, especially with cross-inhibition (data not shown). We conclude that *pta/ackA* strains

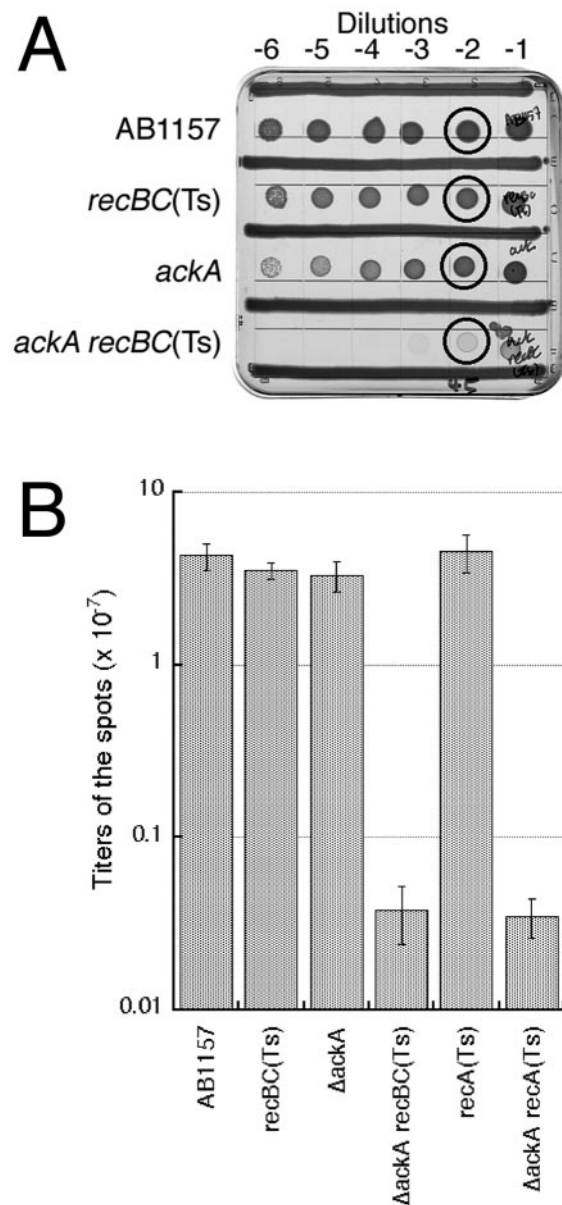


FIG. 7. Quantification of *ackA recBC*(Ts) and *ackA recA*(Ts) inhibition relative to wild-type or single mutant strains. A. A representative plate to show the spots that were taken out for the analysis (circled). The strains are as follows: for *recBC*(Ts), SK129; for *ackA*, IYS4; for *ackA recBC*(Ts), IYS6. B. The titers of CFU. The data are averages of two to five independent determinations \pm standard errors.

have problems with chromosomes only at high temperature, especially when there are growing cells nearby.

Suppressors of *pta rec* synthetic inhibition. Although AB1157 is a standard wild-type strain for DNA metabolism studies of *E. coli*, it was derived through several mutagenesis rounds (1) and may therefore contain uncharacterized metabolic defects which modify behaviors of other mutations. Indeed, when we introduced Δ *ackA* or Δ *pta* mutants into the less-manipulated MG1655 strain (1), we observed only moderate inhibition in *recA* or *recBCD* mutant variants (data not shown), suggesting that the *pta rec* and *ackA rec* inhibition is

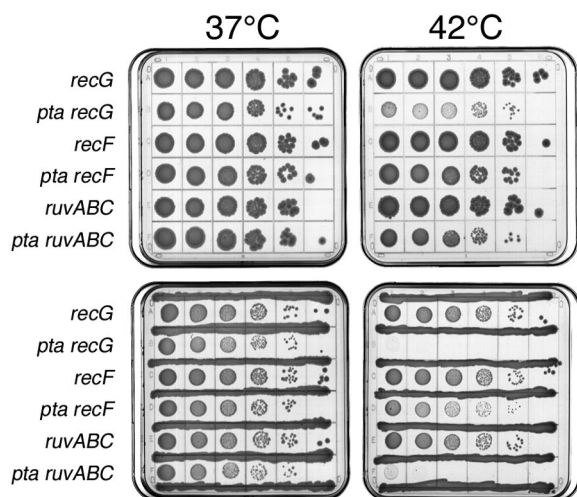


FIG. 8. *pta* mutants are viable in combination with *recF* inactivation but cannot tolerate *recG* or *ruvABC* inactivation at 42°C. Serial 10-fold dilutions of growing cultures were spotted onto plates which were incubated at the indicated temperatures for 36 to 48 h. Where present, cross-inhibiting spots or streaks were those of AB1157. The strains are as follows: for *recG*, N4452; for *pta recG*, JS9; for *recF*, JC9239; for *pta recF*, JS8; for *ruvABC*, AM547; for *pta ruvABC*, JS7.

specific for the AB1157 background. However, since we were interested in the mechanisms of Rec dependence, we could still study them in the AB1157 background, where the inhibition of *ackA rec* and *pta rec* double mutants is strong.

To gain insights into the mechanism of inviability of *pta* mutants, we employed pRL27 mutagenesis to isolate inactivational suppressors which allow *pta rec* mutants to grow under nonpermissive conditions. For this, we carried out the insertional mutagenesis in the *pta recBC*(Ts) mutants and plated the mutagenized cells with cross-inhibition at 42°C (see Materials and Methods). The chromosomal inserts from the colonies that grew under these nonpermissive conditions were P1 transduced into the parental *pta recBC*(Ts) strain and confirmed to suppress its synthetically lethal phenotype. This way, we isolated three weak suppressors, allowing slow growth of the resulting triple mutants (data not shown). We identified positions of inserts in these suppressors by sequencing (Fig. 9). One insert was found in the 583-bp intergenic region between the convergent *yjhR* and *yjhS* genes, the second insert was found to inactivate the *yhhZ* gene, and the third insert inactivated the *yliF* gene; all genes are currently annotated as “function unknown.” The results of the suppressor analysis are considered in Discussion.

DISCUSSION

In a screen for mutants dependent on the RecBCD enzyme in *E. coli*, we tested random Tn5 and pRL27 insertions in the AB1157 *recBC*(Ts) mutant for the inability to grow at 42°C. We isolated two *recBC*-dependent mutations which inactivated *ackA* and *pta*, the genes that form a two-gene operon responsible for the interconversion of acetate and acetyl-CoA. Subsequent experiments with precise deletion-replacement alleles showed that (i) complete inhibition of *ackA rec* and *pta rec* mutants is observed only when they share the same plate with

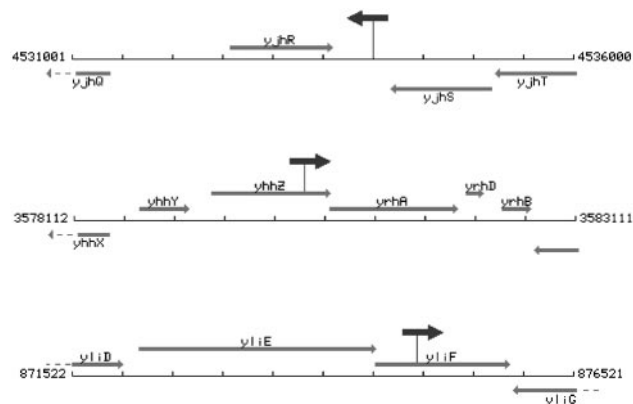


FIG. 9. Position and orientation of pRL27 inserts relative to the chromosomal regions in the suppressor mutants. The vicinity maps of the disrupted gene with exact chromosomal coordinates were generated with the Colibri server (<http://genolist.pasteur.fr/Colibri/genome.cgi>). Open reading frames and their direction are shown by thin gray arrows identified by gene names. The positions of pRL27 inserts are shown by short arrows on sticks, with the orientation of these arrows indicating the orientation of the *kan* gene of the insert.

growing *E. coli* strains (cross-inhibition); (ii) the same effect is emulated by medium modification (yeast extract only, end products of the neighboring metabolic branches); (iii) both *ackA* and *pta* mutants fail to grow in combination with *recA* and *recBC* mutations but show no effect when combined with a *recF* inactivation; (iv) the failure of *ackA rec* and *pta rec* mutants to form colonies under nonpermissive conditions is due to inhibition (extremely slow growth) rather than lethality (loss of colony-forming potential); (v) this synthetic inhibition is more pronounced in the AB1157 background, perhaps due to an uncharacterized additional mutation; and (vi) in contrast to *ackA* mutants, *pta* mutants are also inviable in combination with *ruvABC* and *recG* mutations, suggesting a possible role for acetyl phosphate at late stages of recombinational repair.

The dependence of a mutant on recombinational repair is generally interpreted as an increased level of chromosomal lesions caused by the mutation (5, 19), but the mechanisms leading to chromosomal lesions in *pta* and *ackA* mutants are currently obscure. One (less likely) possibility is that the opposite defects in production of acetyl phosphate (too much acetyl-phosphate in *ackA* mutants and too little acetyl-phosphate in *pta* mutants), which is a known signaling metabolite (27, 42, 44), cause chromosomal lesions via dysregulated signaling. This explanation predicts that the synthetic inhibition of *pta/ackA rec* double mutants will be relieved by inactivation of the target mechanisms of acetyl phosphate signaling. One of the isolated suppressors of the *pta recBC* synthetic inhibition inactivates *yliF*, which codes for a GGDEF family protein of unknown function; the members of this family are widespread in eubacterial genomes and are often found in association with two-component response regulators. The enzymatic reaction catalyzed by these proteins is nucleotide cyclization (13).

Another explanation for the synthetic inhibition of *pta rec* and *ackA rec* mutants is that the inability of *pta* and *ackA* mutants to dump the excess of acetyl-CoA, which is a source of acetyl groups for numerous acetylation enzymes, causes chromosomal lesions via spurious acetylation of DNA-processing

enzymes. This explanation predicts that suppressors of *pta/ackA rec* synthetic inhibition should inactivate acetyltransferases that catalyze this acetylation. Another insertional suppressor of the *pta recBC* inviability that we found in *yhhZ* is just downstream of *yhhY*, which is a putative acetyltransferase of the GNAT family. If the YhhY protein is somehow inactive in our *yhhZ* mutant, this would be consistent with the idea that the excess of acetyl-CoA causes spurious acetylation.

Yet another explanation for the synthetic inhibition of *pta/ackA rec* mutants is that the observed upregulation of the tricarboxylic acid cycle in *pta/ackA* mutants (44) leads to an increase in reactive oxygen species, elevating the background DNA modification. This explanation predicts that suppressors of *pta/ackA rec* synthetic inhibition may slow down the tricarboxylic acid cycle or inactivate DNA repair activities that remove modified DNA bases, thus interfering with DNA replication. The third suppressor insert landed in the intergenic region between *yjhR* and *yjhS*. *yjhR*, a gene likely to be affected by the insertion due to the antisense effect of the kanamycin resistance gene expression, codes for a putative DNA helicase of superfamily I, to which RecB and RecD helicases (components of the RecBCD enzyme) also belong (15). Thus, *yjhR* suppressor is compatible with the DNA modification or repair explanation. On the other hand, *yjhS* codes for a conserved hypothetical protein which is highly related (e-102) to a protein from a cryptic prophage, CP-933P of *E. coli* O157:H7 EDL933. Interestingly, among the distant homologs of YjhS is a probable acetyl xylan esterase, *AxeA*, with a possible function in polysaccharide degradation ($2e-04$) and, with a very low score (8.0), a putative *N*-acetyltransferase. In summary, the results of our suppressor analysis are intriguing and suggestive but at this point do not allow us to clearly distinguish among the three possible reasons (spurious acetylation of proteins, signaling defect, or DNA modification or repair) why *ackA/pta rec* double mutants are synthetically inhibited.

Delineating the phenomena of synthetic lethality and synthetic inhibition proved to be the main crux of this work. Conventional tools used in previous studies of *recA* synthetic lethals, like quantification of chromosomal fragmentation by pulsed-field gel electrophoresis and suppressor analysis (5, 19, 38), are harder to implement in the case of *ackA* and *pta* mutants. Finding better conditions will allow us to isolate more suppressors, while biochemical characterization of the proteins inactivated by suppressor mutations should provide insights into the mechanisms involved in conditional recombinational repair dependence of *pta* and *ackA* mutants.

ACKNOWLEDGMENTS

We are indebted to Jill S. Bradshaw for isolation of the original *pta::Tn5* insertion mutant and for construction of the initial *Δpta::cam* allele and to Paula Welander for isolation of the original *ackA::pRL27* mutant. We thank Alvin John Clark, Ichizo Kobayashi, Richard Kolodner, Sydney Kushner, Robert G. Lloyd, William Metcalf, and Benedicte Michel for providing strains and plasmids and Elena Kouzminova for her help, encouragement, and discussion of results as well as for critical reading of the manuscript.

This work was supported by grant MCB-0196020 from the National Science Foundation.

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