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Do imbalanced nucleotide pools affect holliday junction formation?

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Summary
Holliday junctions are DNA structures necessary for normal cellular maintenance of chromosomes. They are intermediates during homologous recombination and some site-specific recombination reactions, and are vital for DNA repair. This study investigated the occurrence of Holliday junctions in a group of *Escherichia coli* mutants that have imbalanced nucleotide pools due to mutations of genes involved in the biosynthetic or salvage pathways of deoxyribonucleotides. The Segall lab discovered a specific hexapeptide that blocks Holliday junctions and prevents their resolution. This peptide was used in this study. The question being asked was if imbalances in the pools of nucleotides lead to an increased number of Holliday junction intermediates. If that were the case, the group of mutated genes involved in nucleotide or nucleoside biosynthetic or salvage pathways would be hypersensitive to the peptide. I measured the SOS response in the group of mutants. The SOS response is a cellular response to stress such as DNA damage. I wanted to see if they had an increased level of stress after exposure to the peptide and whether the mutations by themselves induced an SOS response. The results indicated that several mutants involved in dNTP synthesis were more sensitive than the wild type to the peptide. My experiments did not answer the question posed in this study; whether nucleotide pools affect Holliday junction formation, but with further research this question could be answered.

Introduction

Inhibiting peptide
A six amino acid long peptide, wrwycr[1], also called peptide d8, was found after screening of synthetic peptide libraries (Boldt et al. 2004) and shown to be bactericidal (Gunderson and Segall 2006). The peptide consists of D-amino acids, which are not found in nature, and unlike L-amino acids not recognized and broken down by *E. coli* cells.

Holliday junctions
Holliday junctions are mobile junctions of four DNA strands (figure 1). Several different proteins are involved in the junction formation and the resolution of the junctions. Holliday junctions occur transiently as intermediates in homologous and site-specific recombination (Gunderson and Segall 2006). These intermediates can also occur during the repair of stalled replication forks resulting from DNA damage. The cysteines of the hexapeptide cause the peptide to dimerize (Boldt *et al*. 2004). It is thought that these dimerized peptides are toxic to bacteria because they block the resolution of Holliday junction formation (Gunderson and Segall 2006).

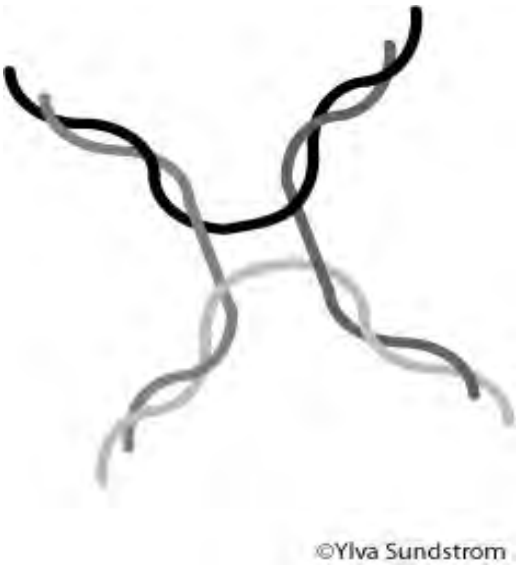

Figure 1. Holliday junction. Two double-helix strands of DNA form a junction.

Mutants sensitive to peptide d8
The Keio collection is a library of systematically mutated *E. coli.* A kanamycin resistance gene was used to replace the contents of each open reading frame, resulting in more than 3900 different mutations.

All mutants from the Keio collection were tested for hypersensitivity by a member of the lab (data not published). 28 different mutants that showed hypersensitivity are involved in nucleotide or nucleoside synthesis. An imbalance of the nucleotide pools could be responsible for the hypersensitivity to peptide d8. Imbalanced pools of nucleotides may slow or stall replication

[1] Tryptophan-Arginine-Tryptophan-Tyrosine-Cysteine-Arginine

forks, which would lead to a higher level of DNA repair intermediates, which briefly form the transient Holliday junctions in the repair process of DNA. When peptide d8 binds the Holliday junction structure, resolvases can not resolve the structure (Gunderson and Segall 2006). This prevents completion of DNA repair.

Stalled junctions
Cells must have nucleic acid building blocks in order to replicate. Mutants of nucleoside and nucleotide synthesis could have imbalanced pools of nucleotides. If some nucleotides are not available, or if they can not be synthesized, the replication process will stall. Stalled replication forks can form four way DNA junctions like Holliday junctions (Figure 1), which are the target for peptide d8 (Gunderson and Segall 2006).

Mutant sensitivity screening
The member of the lab who had previously screened the entire collection for hypersensitivity to the d8 peptide found that several of the sensitive mutants were involved in either de-novo synthesis or salvage pathways of dNTPs were hypersensitive to the peptide.

One way of screening mutants for sensitivity to the peptide in this study was to use minimum inhibitory concentration (MIC). MIC was the lowest concentration of a toxin that was needed to inhibit all growth of bacteria under a certain set of conditions. This was tested by measuring if the optical density changed in cell cultures. If growth was inhibited the optical density remained the same after 24h. By exposing cells to a range of concentrations of a toxin the MIC was determined.

Flow cytometry
Flow cytometry is a method used to analyze each cell in a sample with respect to its size and granularity. A flow cytometer is used to analyze every cell of a liquid sample. The machine shines a light beam on a fine stream of the liquid of the sample. Any particle present in the sample will scatter the beam of light, which is picked up by sensors. A sensor that is in line with the light beam gives a value called forward scatter (FSC), which relates to the size of that particular particle. Sensors perpendicular to the light beam can sense side scattering (SSC), which is related to its shape and size, also called the granularity of a cell. There are also sensors that can detect the presence of fluorescent particles. Detection of fluorescence can be used to see if a certain gene is turned on in each cell by inserting a plasmid or a gene expressing a fluorescent protein. If the gene coding for a fluorescent protein is fused with a promoter of a gene, fluorescence indicates that the specific promoter is activated.

Flow cytometry was used in this study to look at the SOS response. The Keio mutations were inserted into cells with a genetic marker expressing fluorescent proteins fused to a gene that were expressed during the SOS response.

Aims
The aim of this study was to see if Holliday junctions are more frequent in *E. coli* MG 1655 cells that lack some gene involved in nucleotide or nucleoside synthesis. My hypothesis proposed that an imbalance of nucleotide pools would increase the number of stalled replication forks, giving rise to an excess of Holliday junctions where peptide d8 bind. Peptide d8 was used to look for

mutants that were sensitive to the inability to resolve Holliday Junctions.

By testing for sensitivity to other DNA damaging agents, I could determine if the mutants have difficulties in repairing other types of DNA damage. If the mutants show sensitivity to other DNA damaging agents I could conclude that stalled replication forks were not the only reason for hypersensitivity to d8.

The different mutants sensitivity to d8 could varied in different media, due to nutrients, nucleotides or nucleotide precursors available in the media. Growth rates could also have affected the occurrence of Holliday junctions, if the replication mechanism was slower. This was studied by observing the ability of different mutants to grow and by testing the sensitivity to d8 in different defined growth media.

The SOS response activates cellular DNA repair system. I studied whether the mutants were suffering from excess DNA damage, by looking at the level of SOS response that was activated by damage caused by nucleotide imbalance and the peptide.

Results

Peptide hypersensitivity tests
I examined a small subset of the Keio collection (Baba *et al* 2006). In an initial screen by another lab member (data not published) several Keio mutants appeared to be both hypersensitive to the peptide and involved in either de-novo synthesis or salvage pathways of dNTPs.

The peptide sensitivity tests I performed were to determine if the different Keio mutants from the initial screen were hypersensitive to peptide d8. The tests were performed in Mueller Hinton broth (MHB). This is a rich growth medium, commonly used for antibiotic susceptibility tests. It consists partly of organic matter, which is not precisely chemically defined. The tests were performed in microtiter plates. Optical density (OD) readings in a plate reader gave growth curves that were used to determine level of sensitivity to d8.

Table 1 shows the sensitivity of the strains to peptide d8. Each strain was exposed to a range of concentrations of d8. The minimum inhibitory concentration (MIC) was determined for each strain. The mutant was considered sensitive to d8 if the MIC differed between the Keio background strain and the mutant. The Keio background strain had a MIC of 34 μ M d8. Some mutants had MIC of 16 μM d8, and were therefore considered sensitive. One example of MIC differing between a mutant and WT is seen in Figure 2 A and B. A wild type cell culture can recover after exposure to an intermediate dose of the peptide, but sometimes the recovery happens after a longer lag phase. A longer lag indicates difficulties in this recovery. Some mutants showed a consistent longer lag than wild type in recovering from d8. A ratio was calculated from the time it took for each strain to reach 50 % of final OD. If the ratio was 1.14 or higher, the mutant was considered sensitive. The differences in ratio were mainly due to varying lag times after peptide exposure. Figure 2 C show wildtype and the *aegA* mutant under normal growth, and figure 2 D show the longer lag phase of the *aegA* mutant than the wild type when exposed to 16 μM peptide d8.

Table 1. Sensitivity of mutants to peptide d8

Gene mutation	Gene product	MIC [a] of peptide d8	Relative growth ratio[b]	Sensitivity to d8 [c]
None	Keio collection background strain[d]	34 μM	n/a	n/a
yggV	Xanthosine/inosine triphosph pyrophosphatase	16 μM	n/a	yes
nudB	dATP pyrophosphohydrolase NudB	34 μM	0.76	no
dcm	DNA-cytosine methyltransferase	34 μM	0.77	no
mtn	5'-methylthioadenosine nucleosidase / S-adenosyl-homocysteine nucleosidase	16 μM	n/a	yes
rihA	Inosine-uridine preferring nucleoside hydrolase	34 μM	1.68	yes

carB	Carbamoyl-phosphate syntha large chain	16 μM	n/a	yes
cmk	Cytidylate kinase	34 μM	1.50	no
apaH	Bis(5'-nucleosyl)-tetraphosphatase	34 μM	1.08	no
purN	Phosphoribosylglycinamide formyltransferase	34 μM	1.24	yes
purU	Formyltetrahydrofolate deformylase	34 μM	0.99	yes
cpdB	2',3'-cyclic-nucleotide 2'-phosphodiesterase	34 μM	1.12	yes
pyrI	Aspartate carbamoyltransfera regulatory chain	16 μM	n/a	yes
deoC	Deoxyribose-phosphate aldol	34 μM	1.00	no
deoA	Thymidine phosphorylase	34 μM	0.79	no
deoB	Phosphopentomutase	34 μM	0.68	no
deoD	Purine nucleoside phosphorylase	34 μM	1.13	no
aegA	Pyridine nucleotide-disulphic oxidoreductase family protein	34 μM	1.00	yes
surE	5'-nucleotidase	34 μM	0.95	no
pnp	Purine nucleoside phosphorylase	34 μM	1.18	yes
yaiL	Nucleoprotein/polynucleotide associated enzyme	34 μM	0.96	no
tag	DNA-3-methyladenine glycosylase	34 μM	1.00	no
apt	Adenine phosphoribosyltransferase	34 μM	1.11	no
udp	Uridine phosphorylase	34 μM	0.95 [c]	no [c]
ndk	Nucleoside diphosphate kinas	34 μM	1.14	yes
pcnB	Poly(A) polymerase	34 μM	1.19	yes
yjiA	Putative GTPases	34 μM	0.87	no
nrdF	Ribonucleotide reductase of class Ib (aerobic), beta subun	34 μM	0.98	no
tdk	Thymidine kinase	34 μM	1.72	yes

[a] MIC, minimal inhibitory concentration

[b] The relative growth ratio was determined as the ratio between the time to reach 50 % of final OD for WT and mutant. The ratio determined only for mutants with the same inhibitory concentration as the WT. n/a, not applicable.

[c] A mutant was considered sensitive if it had a lower MIC than WT or if the relative growth ratio was 1.14 or higher.

[d] *E. coli* K12 BW25113 *rrnB3 ΔlacZ4787 hsdR514* Δ(*araBAD*)568 *rph*-1

[e] Repeatedly inconsistent data

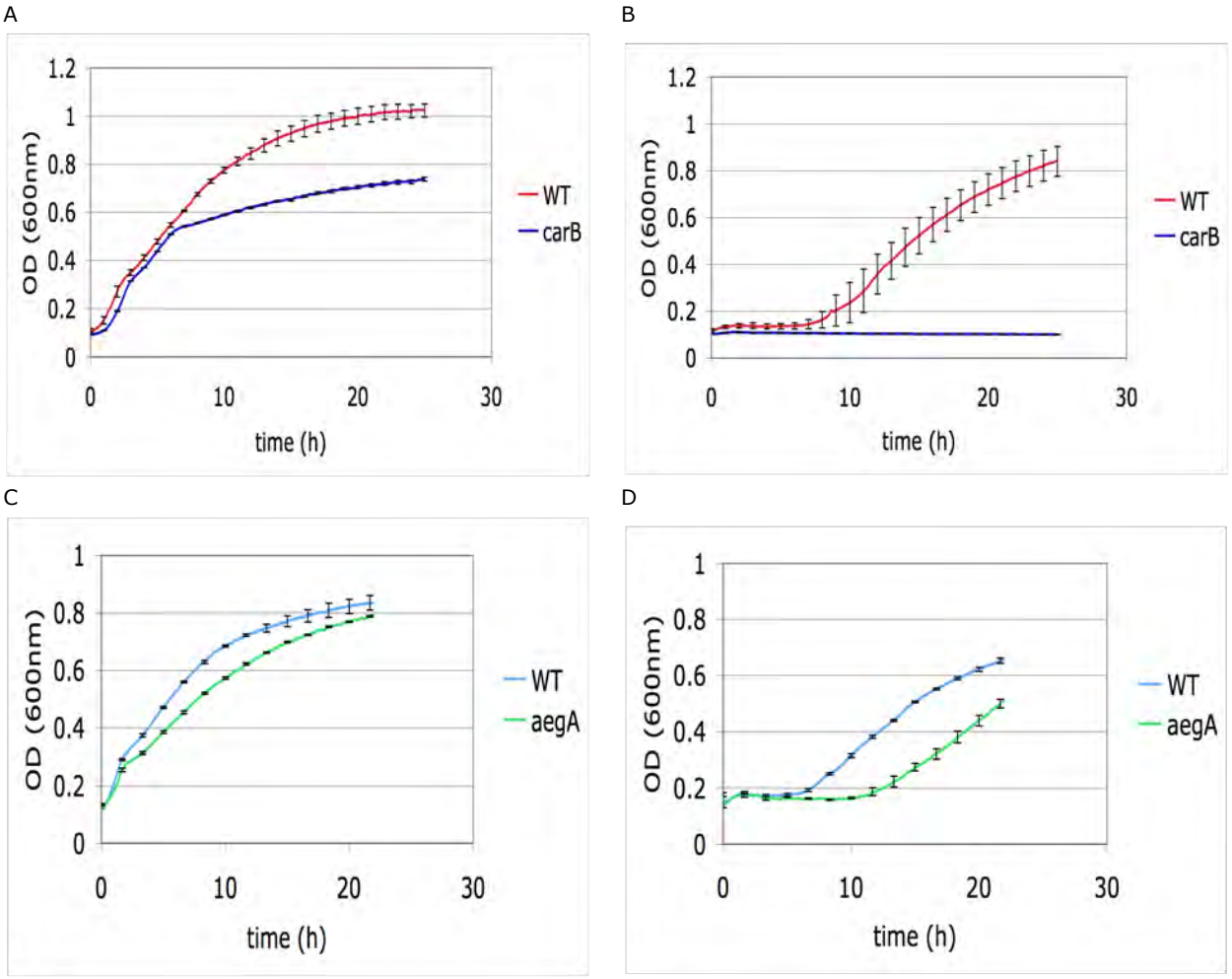

Figure 2 Examples of growth curves used to create table 1. A, growth of the WT strain and *carB* mutant in the absence of peptide d8. B, growth of the same strains in the presence of 16 μM d8. C, growth of untreated wild type and *aegA* mutants; D growth of the same strains in the presence of 16 μM d8. The bars show the standard error for each experiment. 3 independent colonies were used to create each graph. All growth curve experiments were repeated on at least two separate occasions (data not shown).

The mutations from the Keio collection strains were transduced into *E. coli* MG1655, and the resulting strains were retested for peptide d8 sensitivity. Some of the mutations that seemed to cause a longer recovery time after d8 treatment in the Keio collection did not result in the same hypersensitive phenotype when moved into the MG1655 strain. The variability within each experiment was lower in the new strains. The mutations that resulted in sensitivity to d8 in the Keio background strain are listed in Table 2, and can be compared to the sensitivity to peptide d8 after transduction of the mutation into MG1655. These MG1655 strains were used in all sensitivity experiments described in this study, except where other strains were constructed as described. MICs for all mutants in table 2 were 34 μM d8.

Table 2. Sensitivity of mutants in different genetic background strains

Gene mutation	Gene product	Sensitivity to d8[a]	
		Keio background	MG1655 background
yggV	Xanthosine/inosine triphosphate pyrophosphatase	yes	no
mtn	5'-methylthioadenosine nucleosidase / S-adenosylhomocysteine nucleosidase	yes	yes
rihA	Inosine-uridine preferring nucleoside hydrolase	yes	no
carB	Carbamoyl-phosphate synthase	yes [b]	yes [b]
aegA	Pyridine nucleotide-disulphide oxidoreductase family protein	yes	no
purN	Phosphoribosylglycinamide formyltransferase	yes	yes
purU	Formyltetrahydrofolate deformylase	yes	no
cpdB	2',3'-cyclic-nucleotide 2'-phosphodiesterase	yes	yes
pyrI	Aspartate carbamoyltransferase regulatory chain	yes	no
pnp	Purine nucleoside phosphorylase	yes	yes
ndk	Nucleoside diphosphate kinase	yes	yes
pcnB	Poly(A) polymerase	yes	yes
tdk	Thymidine kinase	yes	yes

[a] Sensitivity was determined by comparing relative growth rates by determining the ratio between the time it took to reach 50 % of final OD for the background strain and the mutant. A strain was considered sensitive here if the ratio was 1.14 or higher.
[b] Addition of uridine was necessary for growth

The difference of d8 sensitivity in the original Keio strains and the transduced strains is illustrated in Figure 3. It shows that the *aegA* strain was not more sensitive to peptide d8 than MG1655. This can be compared to Figure 2d, where *aegA* did not recover from d8 exposure as rapidly as the Keio background strain.

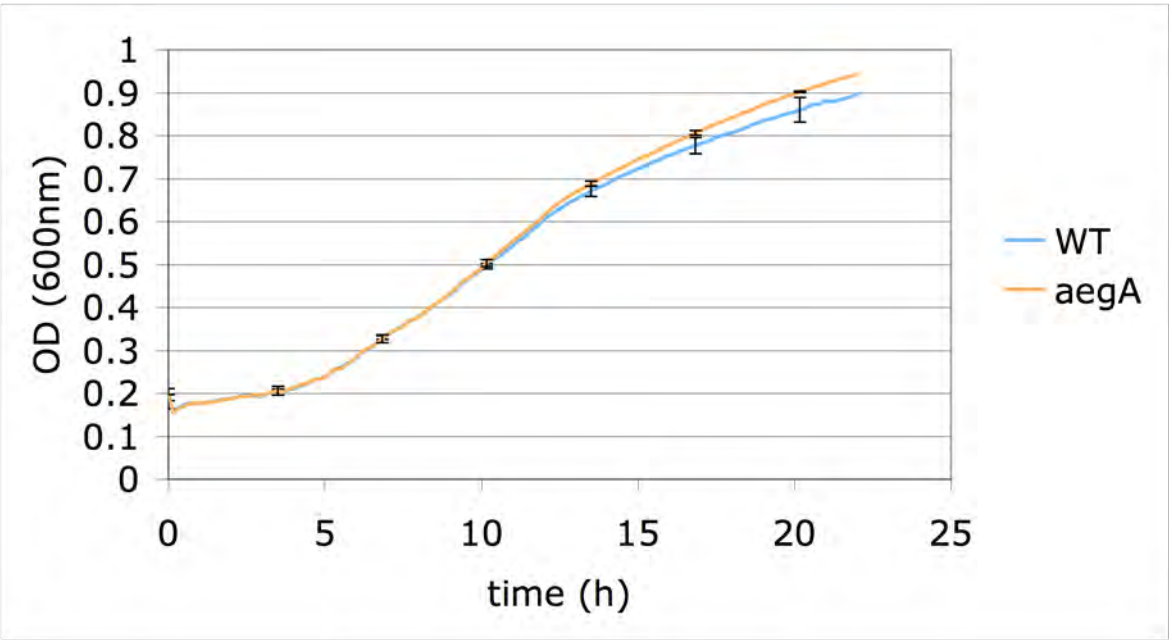

Figure 3. Growth of *AegA* mutant in the MG1655 background. Cells were grown in MHB with 16 μM d8. Error bars show standard error of three independent colonies of each strain.

Sensitivity tests in a defined medium

I wanted to see if the growth medium affected the sensitivity to peptide d8. I used a defined but relatively rich medium containing salts, glucose, casamino acids and vitamins (NGCV). A comparison between sensitivity levels in different types of media could indicate whether the presence of certain nutrients in the medium plays a role to d8 sensitivity.

All strains that were hypersensitive to d8 in the *E. coli* MG1655 background were tested in the NGCV medium. The MIC was 16 μM d8 for all strains that grew in this medium. The growth curves of the cultures that were treated with 8 μM d8 are shown in figure 4 b and can be compared to the untreated cultures in figure 4 a. Since the growth curves looked very different among themselves it was difficult to visually determine in a graph what strains were affected by the peptide in comparison to the MG1655 strain (WT in the graphs).

The relative growth ratio in this medium was determined in the same way as for table 1. Table 3 shows the relative growth ratios. The *carB* mutant had the highest relative growth ratio at 3.25, and the *tdk* and *pnp* mutants had growth ratios above 1.5. Relative growth ratios higher than 1.5 were considered sensitive in this test. This indicates that the *carB* mutant had the highest sensitivity to peptide d8 in this medium, and the *tdk* and *pnp* mutants were also sensitive to d8 in this medium.

All of the mutants as well as the wild type were more sensitive to peptide d8 in this medum than in MHB medium, since the MIC of the same strains in MHB media was 34 μM d8.

Some of the mutants that were more sensitive to peptide d8 than the wild type in MHB medium were not more sensitive than the wild type when grown in NGCV medium. The *pcnB, nkd* and *mtn* mutants were not more sensitive to d8 than the wild type in NGCV medium.

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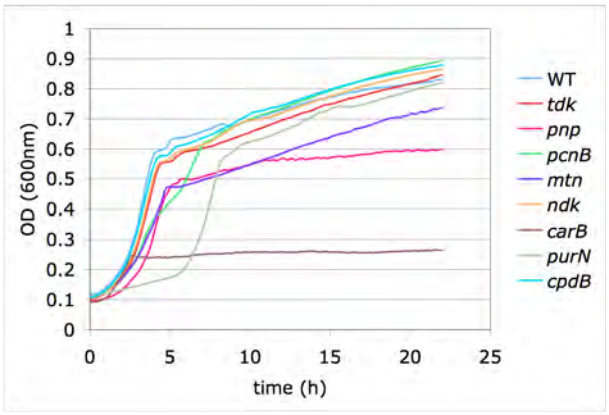

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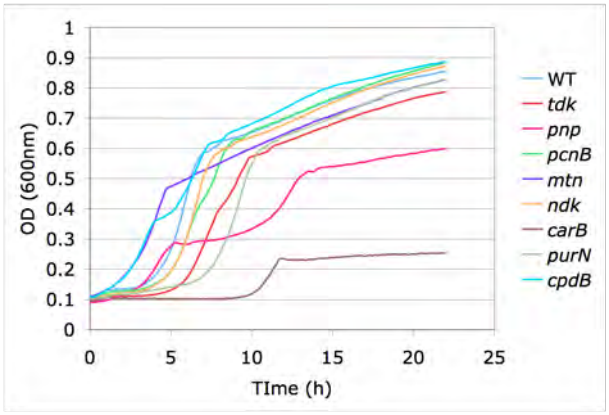


Figure 4. Growth of the different MG1655 mutants in NGCV medium. A, growth in the absence of d8 in NGCV medium; B, growth with 2 μM d8 in NGCV medium.The data comes from one experiment, with each data point being the average from two independent colonies.

Table 3. Growth inhibition of mutants due to peptide d8 in NGCV medium

Gene mutation	Protein	Relative growth ratio [a]
tdk	Thymidine kinase	1.67
pnp	Purine nucleoside phosphorylase	1.56
pcnB	Poly(A) polymerase	0.63
mtn	5'-methylthioadenosine nucleosidase / S-adenosylhomocysteine nucleosidase	0.06
ndk	Nucleoside diphosphate kinase	1.12
carB	Carbamoyl-phosphate synthase	3.25
purN	Phosphoribosylglycinamide formyltransferase	0.63
cpdB	2',3'-cyclic-nucleotide 2'-phosphodiesterase	0.81

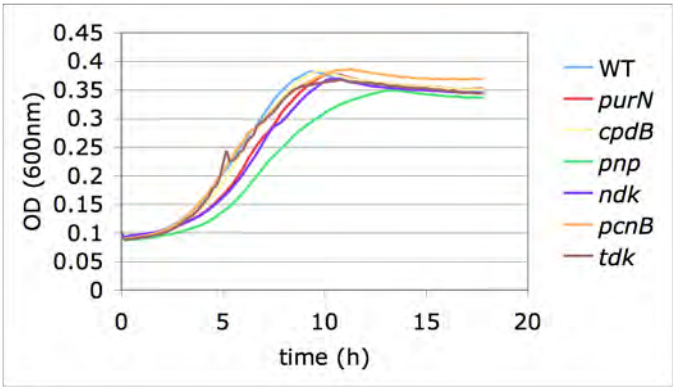
[a] ratio between the time it took for the wildtype vs the mutant strain to reach 50 % of its final optical density

Hypersensitivity tests to d8 in minimal medium

A medium with only glucose as a carbon source does not contain any precursors to nucleotides, such as amino acids and vitamins, which are abundant in NGCV medium. The minimal salts and glucose (NG) medium was used to test whether the lack of amino acids and vitamins had any effects on peptide sensitivity. The MIC for all strains tested in this medium was only 4 μM d8. Figure 5 a show the growth curves of the different strains in NG medium. Figure 5 b show the growth curves of the strains when exposed to 2 μM d8. Figure 5 b displays the longer lag phase of the peptide treated samples. The relative growth ratio was calculated in these conditions, and is shown in table 4. Growth was poor for all mutants as well as the wild type in this medium. The total growth of the MG1655 WT as seen by the total change in OD in figure 5 a was about half of that seen in NGCV (figure 4 a). The *carB* mutant did not grow at all in this medium. The *carB* mutant did not grow in MHB without addition of uridine, and grew poorly in the NGCV medium. Uridine addition did not permit the growth of *carB* in the NG medium. The *mtn* mutant did not grow very well in this medium.

Though the overall growth was poor in this medium, the mutants did not show as much sensitivity to d8 as they did in the richer media. Table 4 shows the relative growth ratios. The *pnp* and *pcnB* mutants show the highest sensitivity to d8 in this medium.

A



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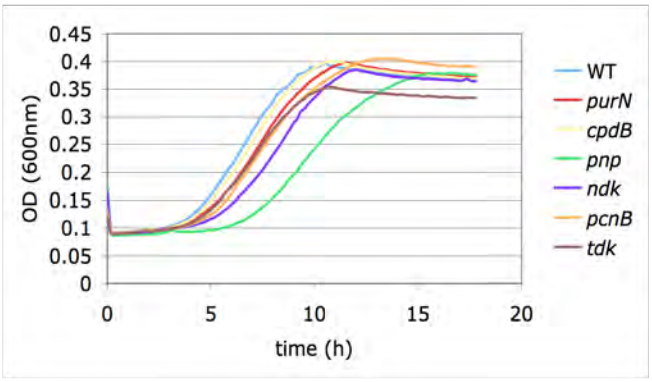


Figure 5. Growth of the different MG1655 mutants in NG medium. A, growth without d8; B, with 8 µM d8. These concentrations of d8 were the highest from which all strains recovered. The data comes from one experiment, with each data point being the average from two independent colonies.

Table 4. Growth inhibition of mutants due to peptide d8 in NG

Gene mutation	Protein	Relative growth ratio [a]
None (wt)		1.17
tdk	Thymidine kinase	1.14
pnp	Purine nucleoside phosphorylase	2.42
pcnB	Poly(A) polymerase	1.85
ndk	Nucleoside diphosphate kinase	1.43
purN	Phosphoribosylglycinamide formyltransferase	0.71
cpdB	2',3'-cyclic-nucleotide 2'-phosphodiesterase	0.85

[a] ratio between the time it took for the wildtype vs the mutant strain to reach 50 % of its final optical density

Sensitivity to other DNA damaging agents

All strains that were sensitive to peptide d8 were further tested for their sensitivity to other DNA damaging agents, namely UV and hydroxyurea. The level of UV sensitivity was quantified and

the level of sensitivity of the different mutants can be seen in Figure 6 a. Mutants defective in the *pnp, ndk, mtn*, and *purN* genes were hypersensitive to the DNA damage caused by UV.

Hydroxyurea sensitivity was measured in all strains sensitive to d8 (table 2). Only the *mtn* and *tdk* mutants showed hypersensitivity to hydroxyurea. They are graphed in Figure 6 b.

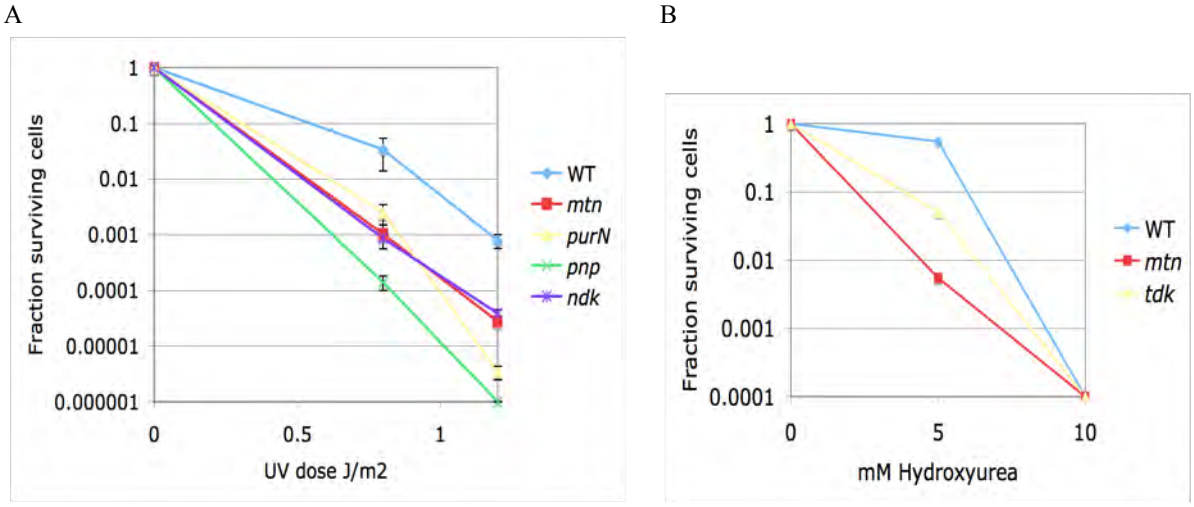


Figure 6. Fraction of surviving cells after exposure to DNA damaging agents. Only results with mutants that were more sensitive to the damaging agent than the wild type are displayed. The WT was MG1655. A, UV sensitivity of cultures plated on MHB agar. Average values of four independent colonies tested on two separate occasions. The error bars are standard deviation. To avoid photoreactivation, the agar plates were handled and incubated in the dark. B, sensitivity to hydroxyurea. Different concentrations of hydroxyurea was mixed in the LB agar. Cultures were plated and colonies were counted. The test was performed once only.

SOS response

The SOS response is activated when DNA is damaged. It results in a number of genes being activated in order to repair the DNA damage. The repair of DNA involves the formation of Holliday junctions. I looked at the expression of SOS response genes, to see if the different mutations led to an increase in Holliday junctions in the presence of d8.

The level of SOS response was tested in two ways. First, the Keio collection gene mutations listed in table 2 were transduced into a strain containing the *sulAp*::mCherry fusion on a plasmid. The *sulA* gene is one of the genes that are turned on in the SOS response. The *sulAp*::mCherry fusion expressed the fluorescent mCherry protein when *sulA* was activated (Janion 2008). The number of cells expressing the mCherry protein in each sample was determined by flow cytometry. Figure 7 shows the percentage of cells expressing mCherry.

None of the untreated mutant strains displayed levels of the SOS response higher than the isogenic parent strain, called WT in figure 7. In the untreated samples between 0.15% and 0.5% of the cells expressed the mCherry protein. All strains tested showed a heightened SOS response, similar to the wild type strain. The *purN* mutant had significantly higher expression levels of mCherry than all other strains. This indicates that it has a higher level of SOS response than the other strains, which could mean that *purN* mutants have more metabolic problems than the other strains. The experiment was only performed once, using three independent cultures of each strain.

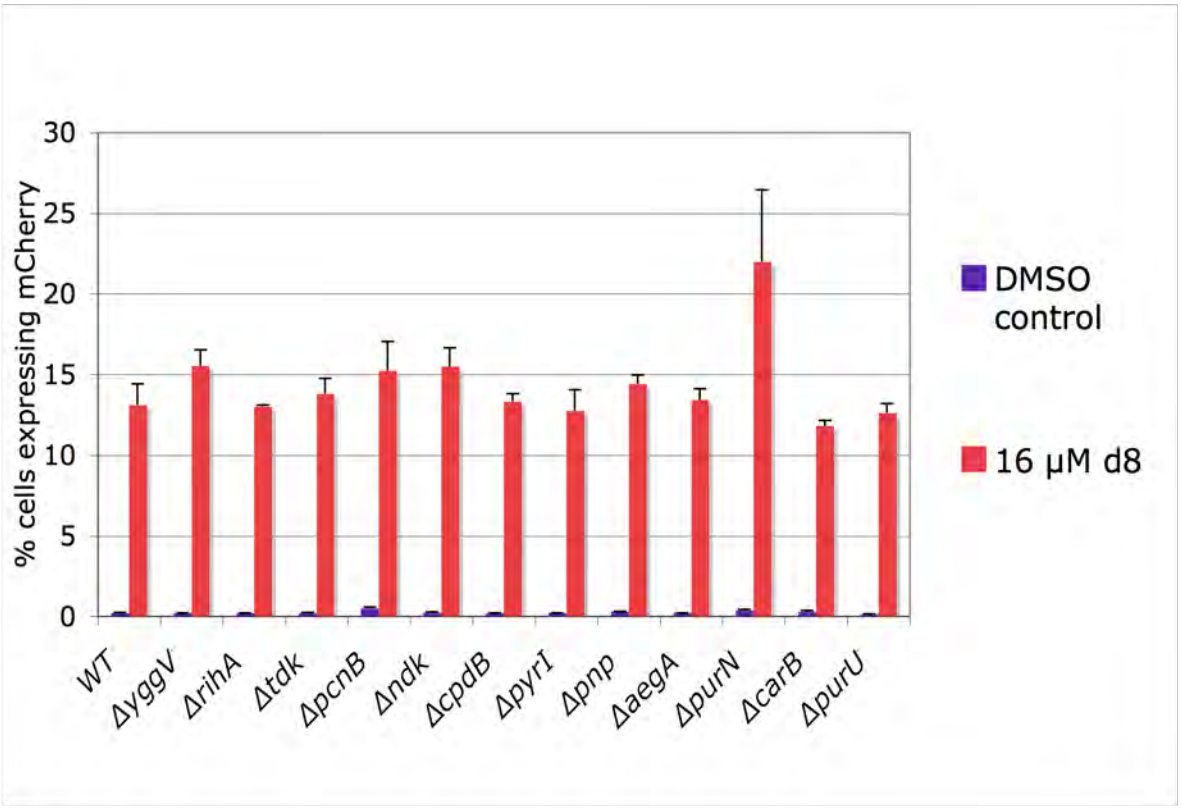
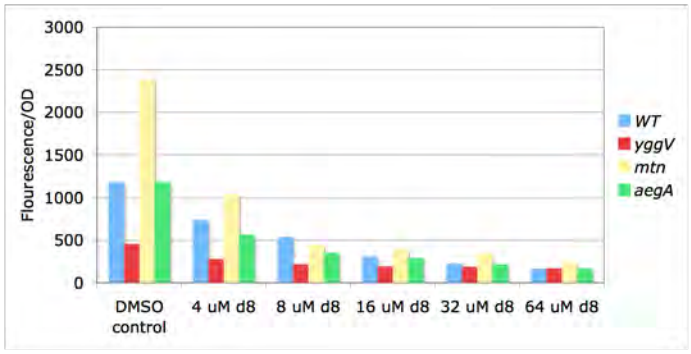


Figure 7. Expression levels of SOS response genes. Mutations were transduced into a *sulAp*::mCherry strain that express the fluorescent mCherry protein when the SOS response is activated. Growth medium was MHB. Columns show percent cells of each sample tested expressing mCherry, as seen during flow cytometry. Error bars indicate standard error.

In a second way to test the SOS response the gene mutations were moved into a *dinD*::*lacZ* fusion strain by transduction. The β -galactosidase-producing gene segment Mu d(*lacZ* Amp^R) was transduced via a phage into the *E. coli* chromosome. The insertion was selected for by plating the transductants on MHB agar with ampicillin. The *dinD* gene was activated in response to DNA damage as part of the SOS response, and β-galactosidase was then expressed.

The test measured the level of β-galactosidase present in each sample. Since the d8 treatment prevents growth as well as expression of some cells, I divided the β-galactosidase values with the optical density values of each sample to get a relative value of β-galactosidase per cell mass unit. These values are graphed in Figure 8.

A



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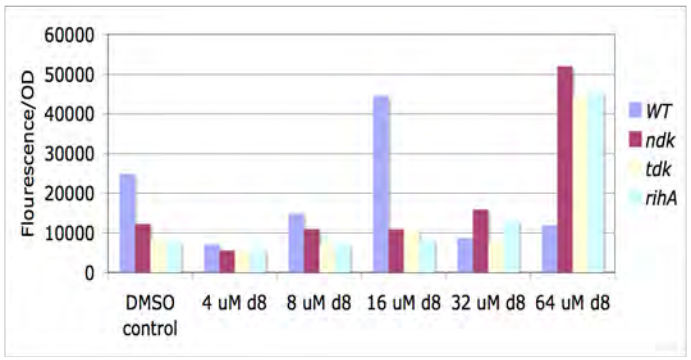

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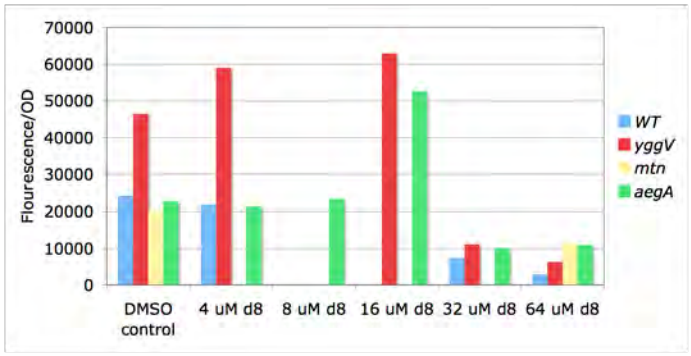

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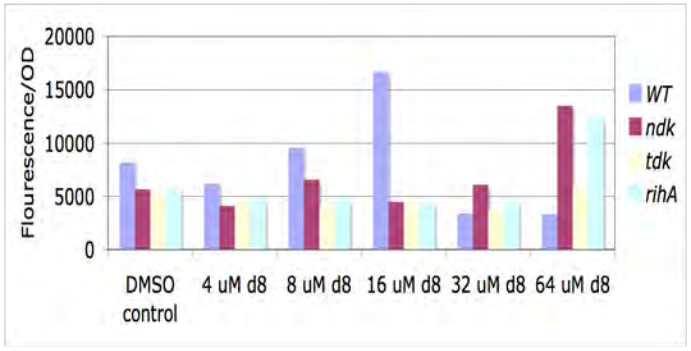

Figure 8. SOS gene expression at 1.5 h after d8 treatment (a and b) and 3 hours after d8 treatment (c and d) The strain called WT was the MG1655 *dinD*::*lacZ* control strain that was used to assess normal levels of *lacZ* expression. To account for the different amounts of cells present at the different time points after treatment the fluorescence values were divided by the optical density of each sample. The values shown are relative units of fluorescence expressed per cell density unit. The experiment was only performed on one day.

The MG1655 *dinD::lacZ* control strain showed an increasing SOS response with increasing doses of the peptide in figure 8 b and d. Comparison of the graphs at 1.5 h (a and b) to the graphs at 3 h (c and d) show that the units were a lot higher at the 3 h measurements than at 1.5 h time point. Most of the mutants tested showed a relatively constant level of SOS response after 1.5 h with increasing doses of d8 treatment, as seen in Figure 8 a and b. The *mtn* mutant had both high basal levels of *dinD* transcription and higher levels of SOS response than the control strain after treatment with different concentrations of d8 as seen in figure 8 a. At the 3 h time point, the *mtn* mutant did not show any increased response, neither for the untreated nor the treated samples. The *yggV* mutant showed lower values than the wild type for both untreated and treated samples at 1.5 h, but showed higher SOS response expression levels than WT for both treated and untreated samples at the 3 h time point. The results of this assay is difficult to interpret since the results were not confirmed, and the assay does not show consistent results between the 1.5 h and the 3 h time points. I would have expected to see similar levels of expression for the untreated samples at both time points. I would also have expected the scale on the y axis of figure 8 a and b to be the same as that of figure 8 c and d, since the same methods were used for the two assays, though they were performed on different days. The results are also not consistently increasing or decreasing with the increasing dose of d8. The experiment was not repeated.

Discussion

Effect of the growth medium

Some mutants were observed to be deficient in growth when the nucleotides were not readily available. This could be an indication that the nucleotide pools were imbalanced, but this sensitivity should be reversed if all nucleotides were readily available in the growth media. The *pnp* and *pcnB* mutants showed the highest level of sensitivity to d8 in NG medium. Since NG medium had glucose as its only carbon source, and the cells needed to produce their own nucleotides to use for growth, the result may indicate that the the *pnp* and *pcnB* mutants created more Holliday junctions or stalled replication forks in this media. This could be because the mutants had imbalanced nucleotide pools in this medium, which was my hypothesis.

In NGCV medium lots of building blocks were provided. *carB, tdk* and *pnp* mutants had a growth ratio higher then 1.5. It is known that *mtn* is auxotrophic for biotin (Choi-Rhee and Cronan 2005), and *carB* is involved in the early pathways of arginine and uridine synthesis. I expected the nucleotide pools of all mutants to be more balanced in NGCV media than in NG media, since there were more nucleotides and nucleotide precursors in this media. According to my hypothesis, the mutants *pnp* and *pcnB* should not be as sensitive to the peptide as they were in the NG medium if the nucleotide pools were balanced in his medium. If the *pnp* and *pcnB* mutants still has imbalanced nucleotide pools in this medium, I would expect them to be sensitive to the peptide.

It took longer time for all strains, including the wild type to reach maximum cell density in the minimal NG medium than in the NCV medium. The slower growth rates could account for fewer replication forks present at any time (Nordman 2007). Slower growth could give the cell more time to recover from the peptide, and result in fewer mistakes in the replication process. Fewer mistakes in replication would mean fewer Holliday junctions formed, and less sensitivity to peptide d8. This could be one reason why fewer mutants are sensitive to the peptide in the NG and NGCV media than in the rich MH broth. It is a small numerical difference between the growth curves of the mutants that are considered sensitive and those that are not considered sensitive in this project. The small differences make it very important to repeat all assays to get statistically relevant differences in the numbers before any definitive conclusions can be made.

DNA damaging agents

The UV and HU tests show each mutant's ability to recover from DNA damaging agents. When an *E. coli* is exposed to any type of DNA damage, the SOS response is activated. The SOS response could be affected by a mutation. A mutation can cause for example a heightened constitutive SOS response (Burger *et al* 2002) or a mutation could lower the efficiency of the SOS response. If the SOS response were affected by any of the mutations I have tested, it would play a role in the mutants' ability to recover from peptide exposure. If some of the mutants have a higher or lower SOS response than others, in addition to them having imbalanced nucleotide pools, the hypothesis that imbalanced nucleotide pools cause a higher frequency of stalled replication forks will need to take the SOS response into account.

UV exposure leads to DNA damage, most commonly in the form of cyclobutane pyrimidine dimers. In daylight these lesions are repaired directly by photolyase (Sharma *et al.* 1982). Since

the samples tested in this study were kept in the dark after UV exposure, I can conclude that the UV sensitive mutants *mtn, purN, pnp* and *ndk* are somewhat deficient in performing the DNA repair in the dark that is necessary after UV exposure. These processes would include recombination events, which generate Holliday junctions. UV irradiation damage is known to stall replication forks (Burger *et al.* 2002). The *mtn*, *purN*, *pnp* and *ndk* strains that are hypersensitive to peptide d8 could be so due to generally unstable replication mechanisms due to the mutations. I do not think that these mutants that are sensitive to UV should be used to determine if imbalanced nucleotide pools affect the formation of Holliday junctions without careful considering that they may have some problems in their replication process.

Hydroxyurea (HU) inhibits deoxyribonucleic acid synthesis by inhibition of the enzyme ribonucleoside diphosphate reductase. This enzyme catalyzes the conversion of all four ribonucleoside diphosphates into corresponding deoxyribonucleoside diphosphates. HU sensitivity tests indicate that cells with deletions of *mtn* or *tdk* are more deficient than the other mutants and the wild type strain in recovering from the inability to transform ribonucleodise diphosphates into deoxyribonucleoside diphosphate. The data from the hydroxyurea tests comes from one test, on one day only, and are therefore not very reliable.

SOS response

The SOS response as measured by *sulA* expression was higher for the *purN* mutant than for the wild type strain used, though the error bars were high, as seen in figure 7. I would like to repeat the assay to test the significance of that result. I would also like to test the *purN* mutant in the *dinD::lacZ* strain used in the second SOS-response assay used (figure 8). The heightened SOS response in the *purN* mutant indicates that the SOS response is affected by the peptide. Since the SOS response is activated, I can assume a higher frequency of holliday junctions in this mutant, and it would be difficult to use this mutant to answer my hypothesis. The results from the *lacZ* expression assay shown in figure 8 are not shown in table 4 since they can not be statistically evaluated without a repeat of the assay due to the big variability in the assay. Overall, both assays used to detect SOS activity indicated a low stress response to the Keio mutations in comparison to that of the wild type. If the SOS response of the mutants is at the same level as the wild type, they can be used to further explore if imbalanced nucleotide pools affect holliday junction formation. Nordman et al. (2007) demonstrated a temporal relationship between the deoxyribonucleotide (dNTP) synthesis and DNA replication in vivo, which could explain why a slow or stalled synthesis of dNTPs could slow down the DNA replication without much of an SOS response. The uneven results of the measurements of the levels of SOS response both in the absence of treatment and after d8 treatment prevents me from drawing any conclusions, but low levels of SOS response could indicate that the imbalances of nucleotides slows the process of growth in the presence of peptide d8, perhaps causing more stalling of forks but not increasing their frequency of collapse.

Table 4. Summary of results.

Mutant	Gene function	Sensitivity				Hightened SOS response[a]
		d8 in NG	d8 in NGCV	UV	HU	
carB	Carbamoyl phosphate synthase	n/a[b]	yes	no	no	no
mtn	5'-methylthioadenosine nucleosidase / S-adenosyl homocysteine nucleosidase	no	no	yes	(yes)[c]	no
ndk	Nucleoside diphosphate kinase	no	no	yes	no	no
pcnB	Poly(A) polymerase	yes	no	no	no	no
pnp	Purine nucleoside phosphorylase	yes	yes	yes	no	no
purN	Phosphoribosyl glycinamide formyltransferase	no	no	yes	no	yes
tdk	Thymidine kinase	no	yes	no	(yes)	no

[a] The SOS response without d8 exposure, data from figure 7. The assay that measured *lacZ* expression, as seen in figure 8 is not included in this table.
[b] Not applicable. The *carB* mutant did not grow in NG media.
[c] Brackets indicate uncertain results, as the test was performed without replicates on only one day.

Holliday junctions and peptide d8

The hypothesis I began to test was that an imbalance of dNTPs would lead to an increase in the number of Holliday junctions in cells and lead to hypersensitivity of these mutants to peptide d8. The way I tested this hypothesis was by searching for mutants that showed hypersensitivity to peptide d8. I discovered that some of the mutants took longer time to recover from exposure to the peptide than the background strain. I expected most of the mutants I initially tested to have imbalanced nucleotide pools, and struggle with DNA replication due to nucleotides not being available. Stalled replication forks are peptide targets. The experimental results of some of the mutants do not support the hypothesis. Table 4 summarizes the results of the different tests performed on the mutants that were sensitive to peptide d8 when inserted in the MG 1655 strain. The UV and hydroxyurea sensitivity tests indicate that some mutants have deficiencies in DNA repair, aside from what was caused directly by d8. The *mtn* and *tdk* mutants were more sensitive to hydroxyurea than the other mutants tested and the wild type. That could be due to imbalanced nucleotide pools. I would expect that mutants with imbalanced nucleotide pools would be sensitive to hydroxyurea. A mutation in a nucleotide or nucleoside synthesis gene does not

necessarily mean that the mutant have unbalanced nucleotide pools since *E. coli* have several alternate ways to acquire nucleotides such as recycling components or using alternate pathways (Burger *et al*. 2002). More tests are needed to find which mutants have imbalanced nucleotide pools that could apply to my hypothesis. I could continue this work by further exploring the *carB, pcnB* and *pnp* mutants, since they do not show sensitivity to UV, and they do not show a heightened basal SOS response, as summarized in table 4, but I would also like to repeat several of the assays described in this study.

I would find it interesting to further investigate the relationship between imbalanced nucleotide pools and Holliday junction formation by determining the rate and amount of nucleotide incorporation into the DNA of the different mutants in the presence or absence of the peptide. One experiment that could validate some of my data in this study would be to determine the ratios and amounts of dNTPs in the different mutated cells. This could be done through high-performance liquid chromatography (Muller *et. al 1995*). If I could determine the level of SOS response and quantify the nucleotide pools, I could get closer to determine if imbalanced nucleotide pools affects holliday junction formation. I still think this hypothesis is plausible after this study, and would like to explore it further.

Materials and Methods

Strains and strain construction
The Keio collection (Baba *et. al* 2006) contains gene mutations, caused by insertion of a kanamycin resistance gene surrounded by flippase recognition targets (FRT) regions. FRT regions are specific sequences homologous to the up- and downstream regions of the targeted gene, which were used in the making of the in-frame, single-gene deletion of mutants (Baba et al. 2006). These FRT flanked kanamycin genes had been systematically inserted in genes located within known open reading frames. These FRT-flanked gene insertions were transduced into *MG1655* via a P1 transduction (described by Davis *et al*. 1980), as described in table 5.

The P1 phage lysates of the Keio mutations carrying kanamycin resistance were also used for P1 transduction into the ampicillin resistant strain *E. coli sulAp*::mCherry strain as described above. The gene encoding the mCherry red fluorescent protein was fused to the *sulA* gene on a plasmid. These transductants were selected for by growth on plates containing both kanamycin and ampicillin.

The Keio mutations were also used for P1 transduction into the *E. coli dinD*::Mu d(*lacZ* ApR) strain. These transductants were selected for by growth on plates containing both kanamycin and ampicillin. Expression of β-galactosidase when the cell is undergoing SOS response is a result of the *din D::lacZ* fusion. I was unable to transduce the mutation of gene *pnp* into the *dinD*::Mu d(*LacZ)* strain by P1 transduction.

Table 5. *E. coli* strains used in this study

Strain	Genotype	source
G817	K12 BW25113 *rrnB3 ΔlacZ4787 hsdR514* Δ(*araBAD*)568 *rph*-1	T. Baba
34-5B	G817 *yggV::* FRT *Kan*^{R} FRT	T. Baba
40-8D	G817 *nudB::* FRT *Kan*^{R} FRT	T. Baba
40-8E	G817 *dcm::* FRT *Kan*^{R} FRT	T. Baba
50-4E	G817 *apaH::* FRT *Kan*^{R} FRT	T. Baba
50-4H	G817 *mtn::* FRT *Kan*^{R} FRT	T. Baba
50-6G	G817 *rihA::* FRT *Kan*^{R} FRT	T. Baba
50-4D	G817 *carB::* FRT *Kan*^{R} FRT	T. Baba
50-7C	G817 *cmk::* FRT *Kan*^{R} FRT	T. Baba
50-4E	G817 *apaH::* FRT *Kan*^{R} FRT	T. Baba
54-4F	G817 *purN::* FRT *Kan*^{R} FRT	T. Baba
50-7F	G817 *purU::* FRT *Kan*^{R} FRT	T. Baba
54-6E	G817 *cpdB::* FRT *Kan*^{R} FRT	T. Baba
54-6H	G817 *pyrI::* FRT *Kan*^{R} FRT	T. Baba
54-7C	G817 *deoC::* FRT *Kan*^{R} FRT	T. Baba
54-7D	G817 *deoA::* FRT *Kan*^{R} FRT	T. Baba
4-8E	G817 *deoB::* FRT *Kan*^{R} FRT	T. Baba
54-7E	G817 *eoD::* FRT *Kan*^{R} FRT	T. Baba
54-9D	G817 *aegA::* FRT *Kan*^{R} FRT	T. Baba
64-5F	G817 *surE::* FRT *Kan*^{R} FRT	T. Baba
80-4E	G817 *pnp::* FRT *Kan*^{R} FRT	T. Baba
80-8F	G817 *yaiL::* FRT *Kan*^{R} FRT	T. Baba
80-10E	G817 *tag::* FRT *Kan*^{R} FRT	T. Baba

86-4E	G817 *apt::* FRT *Kan*^{R} FRT	T. Baba
86-5B	G817 *udp::* FRT *Kan*^{R} FRT	T. Baba
86-5F	G817 *ndk::* FRT *Kan*^{R} FRT	T. Baba
88-7B	G817 *pcnB::* FRT *Kan*^{R} FRT	T. Baba
88-3D	G817 *yjiA::* FRT *Kan*^{R} FRT	T. Baba
54-5D	G817 *nrdF::* FRT *Kan*^{R} FRT	T. Baba
G652	MG1655	S. Maloy
EDT 1878	G652 *yggV*::FRT *Kan*^{R} FRT	this study
EDT 1879	G652 *mtn*::FRT *Kan*^{R} FRT	this study
EDT 1880	G652 *rihA*::FRT *Kan*^{R} FRT	this study
EDT 1881	G652 *carB*::FRT *Kan*^{R} FRT	this study
EDT 1882	G652 *purN*::FRT *Kan*^{R} FRT	this study
EDT 1883	G652 *purU*::FRT *Kan*^{R} FRT	this study
EDT 1884	G652 *cpdB*::FRT *Kan*^{R} FRT	this study
EDT 1885	G652 *pyrI*::FRT *Kan*^{R} FRT	this study
EDT 1886	G652 *aegA*::FRT *Kan*^{R} FRT	this study
EDT 1887	G652 *pnp*::FRT *Kan*^{R} FRT	this study
EDT 1888	G652 *ndk*::FRT *Kan*^{R} FRT	this study
EDT 1889	G652 *pcnB*::FRT *Kan*^{R} FRT	this study
EDT 1890	G652 *tdk*::FRT *Kan*^{R} FRT	this study
G 775	JC13059 *sulAp*::*mCherry* (*Ap*^{R})	S. Sandler
EDT 1865	G775 *yggV*::FRT *Kan*^{R} FRT	this study
EDT 1866	G775 *mtn*::FRT *Kan*^{R} FRT	this study
EDT 1867	G775 *rihA*::FRT *Kan*^{R} FRT	this study
EDT 1868	G775 *carB*::FRT *Kan*^{R} FRT	this study
EDT 1869	G775 *purN*::FRT *Kan*^{R} FRT	this study
EDT 1870	G775 *purU*::FRT *Kan*^{R} FRT	this study
EDT 1871	G775 *cpdB*::FRT *Kan*^{R} FRT	this study
EDT 1872	G775 *pyrI*::FRT *Kan*^{R} FRT	this study
EDT 1873	G775 *aegA*::FRT *Kan*^{R} FRT	this study
EDT 1874	G775 *pnp*::FRT *Kan*^{R} FRT	this study
EDT 1875	G775 *ndk*::FRT *Kan*^{R} FRT	this study
EDT 1876	G775 *pcnB*::FRT *Kan*^{R} FRT	this study
EDT 1877	G775 *tdk*::FRT *Kan*^{R} FRT	this study
G 192	W3110 N99 *dinD*::*Mu d*(*lacZ Ap*^{R})	G. Walker
EDT 1853	G192 *yggV*::FRT *Kan*^{R} FRT	this study
EDT 1854	G192 *mtn*::FRT *Kan*^{R} FRT	this study
EDT 1855	G192 *rihA*::FRT *Kan*^{R} FRT	this study
EDT 1856	G192 *carB*::FRT *Kan*^{R} FRT	this study
EDT 1857	G192 *purN*::FRT *Kan*^{R} FRT	this study
EDT 1858	G192 *purU*::FRT *Kan*^{R} FRT	this study
EDT 1859	G192 *cpdB*::FRT *Kan*^{R} FRT	this study
EDT 1860	G192 *pyrI*::FRT *Kan*^{R} FRT	this study
EDT 1861	G192 *aegA*::FRT *Kan*^{R} FRT	this study
EDT 1862	G192 *ndk*::FRT *Kan*^{R} FRT	this study
EDT 1863	G192 *pcnB*::FRT *Kan*^{R} FRT	this study
EDT 1864	G192 *tdk*::FRT *Kan*^{R} FRT	this study

[a] Strain used by Baba *et al.* in making the Keio collection.

Growth media and agar

Luria-Bertani (LB) broth contained 10 g Bacto tryptone, 5 g Bacto yeast, 5 g NaCl and water, which make 1 L. The LB agar that was used consisted of 15 g (Sigma) agar agar to 1 L LB broth. Agar was used for plating and maintenance of the strains. For selection during strain construction antibiotics kanamycin and ampicillin were added to 50 μg/ml.

Mueller-Hinton broth (MHB) (21 g Difco MHB powder to 1L H_2O), was used for MIC and growth curve assays unless other media were specified. NG medium (Davis *et al*. 1980) is a minimal medium with added glucose (20 ml 50 x NCE salts, 1 ml 1 M $MgSO_4$ and 2 g glucose) that was used in this study. NGCV medium is NG with 1 % casaminoacids, and 1 x of a vitamin mix. The 500 x vitamin mix contains 0.025 % each of folic acid, pantothenic acid, nicotinamide, pyridoxal HCl and thiamine HCl, as well as 0.0025 % riboflavin and 0.05 % biotin. Uridine was added as a nutritional supplement (Davis *et al.* 1980) to final concentrations of 10 μg/ml in the assays involving the *carB* mutant, as indicated.

Peptide
The peptide d8, wrwycr was synthesized by BioSynthesis (Lewisville, Texas) or by Sigma-Genosys (The Woodlands, Texas). Stock solutions of around 10 mM peptide were prepared in the lab. The solvent used was dimethyl-sulfoxide (DMSO). The stock solutions were kept at room temperature to permit the cysteine residues to dimerize. Boldt *et. al.* (2004) determined that the peptide is more efficient as a dimer. The level of dimerization is optimally at 90 % or higher, and can be analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC)

Sensitivity tests.
To test for d8 sensitivity, overnight cultures were grown and sub-cultured at a ratio of 1:100 in 150 μl cultures in microtiter plates. The subcultures were incubated for approximately 1h, to reach early exponential growth phase. The OD_{600} was checked to make sure all strains in the 96-well microtiter plate had the same cell density. If cell density varied within a microtiter plate, dilutions were made so the treatments were given at OD_{600} values between 0.08 and 0.1. d8 was then added, at concentrations of 1, 2, 4, 8, 16, 32 and 64 μM. Optical density readings were taken every 10 minutes for 24 hours.

To test for UV sensitivity, cultures were grown overnight and then subcultured 1:100 in LB. The cell density was measured in a Beckman Coulter UD 640 spectrophotometer until it reached an OD_{640} of 0.4 nm. Dilutions were made in Tris-magnesium (Tris-Mg) (1 mM Tris, pH 7.4 adjusted using HCl, 10 mM $MgCl_2$). Serial dilutions of bacterial cultures were plated on LB agar, and an ULTRA-LUM UVC-515 Ultraviolet multilinker was used to expose the plates to 254 nm UV light. The plates were exposed to 0, 8 and 12 joules / cm^2 before overnight incubation in the dark (Kantor and Deering 1967) at 37 ^oC.

Hydroxyurea (HU) sensitivity was determined by adding 5 mM and 10 mM to LB agar. HU (Calbiochem) was dissolved in melted LB agar, and bacterial growth was assayed on the solid agar containing HU. Bacterial cultures were serially diluted in ten fold steps in a 96 well microtiter plate with Tris-Mg buffer, and plated on the HU containing agar using a 48-prong device.

Optical density and fluorescence readings
Optical densities were measured in a Molecular Devices Spectra Max Plus 96 well plate reader at 600 nm. The software used was SoftMax Pro (Molecular Devices, Sunnyvale California). Growth curves were determined over 24 hours, with readings every 10 minutes at a constant temperature of 37 ^oC. Single time point readings were performed using the same machine and

software. For single time point readings the 96 well plates were incubated at 37 ^{o}C between readings. The fluorescence of samples in a 96 well opaque microtiter plate was read using a Spectra Max Gemini microplate reader (Molecular Devices)

Flow cytometry
Overnight cell cultures of the strain expressing mCherry were grown in MH broth, subcultured 1:100 and incubated at 37 ^{o}C with agitation for 90 minutes or until OD_{640} of 0.4 was reached. d8 peptide was added to a concentration of 16 μM, and the culture incubated under the same conditions for 90 minutes. The flow cytometer was used to measure the levels of mCherry expression using the PE-Texas Red filter, as well as forward scatter (FSC) and side scatter (SSC). FSC scatter plots the size of each cell, SSC plots the granularity of each cell. The software used to analyze data was FACS Diva (X).

4-methyl umbelliferyl β-D-galactosidase assay
Overnight cultures of cells containing the *din D::lacZ* fusion were grown in MH broth. Subcultures were made by diluting these cultures 1:100 in MH broth in a 96 well microtiter plate to a final volume of 150 μL. The subcultures were then incubated at 37 ^{o}C for one hour while shaking, and OD readings were taken. 10 μL aliquots were transferred to an opaque flat bottom 96 well plate, which was sealed and frozen at -80 ^{o}C for at least 10 minutes, never more than 3 days. d8 was added to 16 μM, and the microtiter plate was kept at 37 ^{o}C for a total of 23 h after d8 addition. 10 μL aliquots were taken and immediately frozen, and OD of the 96 well microtiter plate was read at the timepoints 90 minutes, 3 h and 24 h.

The frozen 10 μl aliquots were thawed at room temperature. 1 x buffert (60 mM K_2HPO_4, 40 mM KH_2PO_4 , 100 mM NaCl) and 4-methyl umbelliferyl –D-galactosidase (MUG) dissolved in DMSO was added to a final concentration of 0.4 mM. After 45 minutes a glycine buffer (200 μM adjusted to pH 10.3 using NaOH) was added to stop the reaction between MUG and β-galactosidase.

β-galactosidase forms the fluorescent 4-methylumbelliferone from MUG. The fluorescence was determined in a fluorimeter at 360 nm excitation wavelength and 450 nm emission.

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