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β-lactam antibiotics, *recA* mutation and SOS response

β-laktamski antibiotiki, mutacija *recA* in odziv SOS

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Abstract: The claim that β-lactam antibiotics induce the SOS response, allowing *E. coli* survival in the presence of low antibiotic concentrations, was evaluated. No association between the *recA* gene and antibiotic survival rate was found. Disagreements with published observations are attributed to discrepancy in minimum inhibitory concentrations and growth characteristics of various strains carrying the *recA* mutation. Moreover, β-lactam antibiotics do not induce expression of the SOS regulated gene *cka*, encoding colicin K in wild-type strains.

Keywords: SOS response, *E. coli*, β-lactam antibiotics, antibiotic resistance

Povzetek: Preverili smo trditev, da β-laktamski antibiotiki sprožijo odziv SOS, kar naj bi omogočalo bakteriji *E.coli* preživeti v prisotnosti nizkih koncentracij antibiotikov. Opravljeni poizkusi niso pokazali nobene povezave med delovanjem produkta gena *recA* in stopnjo preživelosti ob prisotnosti antibiotika. Neskladje z objavljenimi opazovanji lahko pripišemo razlikam v minimalni inhibitorni koncentraciji in dramatično različnim lastnostim rasti pri doslej uporabljenih sevih z mutacijo v genu *recA*. Z uporabo genetsko neokrnjenega seva smo ugotovili, da β-laktamski antibiotiki ne sprožijo izražanje gena za kolicin K (*cka*), gena, ki je nadzorovan s sistemom SOS.

Ključne besede: odziv SOS, *E. coli*, β-laktamski antibiotiki, odpornost proti antibiotikom

Introduction

In most bacteria, DNA damage is addressed by a set of genes constituting the SOS response. The response can be triggered by diverse exogenous treatments that elicit DNA damage and physical stress such as high pressure. In addition, stalled replication forks, unrepaired defects following recombination or chromosome segregation, as well as DNA damage caused by metabolic intermediates can also induce the SOS response. The LexA and RecA (recombinase A) proteins are its key regulators (Erill et al. 2007). RecA protein responds to a single-stranded DNA formed at sites of the DNA damage and triggers degradation of LexA transcriptional repressor. As a consequence, a number of LexA repressed genes are induced, covering among others synthesis and secretion of

colicins in E. coli. In 2004, Miller et al. reported that sublethal concentrations of β-lactam antibiotics trigger the SOS response through the DpiBA two-component signal transduction system. Although this effect is generally accepted, several facts question the nature of this induction.. First, the DpiA and DpiB proteins have been shown to be involved in anaerobic citrate metabolism (Kaspar and Bott 2002). DpiB only binds specifically to the promoter regions of *cit* and *mdh* operons involved in citrate metabolism. In addition, it was found that DpiBA may also control the hexuronate dissimilation pathway via the *exuTR* operon, which may possibly be linked to citrate fermentation (Yamamoto et al. 2008). Second, a transcriptome profiling demonstrated that *dpiA* was not induced as one of the SOS genes after exposure to UV, in fact, it was downregulated (Courcelle et al. 2001). Also, contrary to the observation by Miller *et al*., *dpiA* and *dpiB* were found to be downregulated during ampicillin treatment (Sangurdekar et al. 2006).

The aim of this study was to elucidate the mechanism by which arrest of cell-wall synthesis by β-lactam antibiotics triggers the expression of the seemingly unrelated *dpi* operon. However, neither differences in survival rates of *recA* mutants nore β -lactam induction of the SOS response were observed when reproducing certain experiments published by Miller et al. (2004).

Material and Methods

Bacterial strains and plasmids

Parental bacterial strains are listed in Table 1. Strains AB1157 *recA*::Kn, MG1655 *recA*::Kn and RW118 *recA*::Kn were constructed by transducing the respective wild-type strains with a P1 lysate, prepared from strain JW2669 carrying a *recA* deletion mutation, and by selecting for kanamycin resistance. Transductants were **verified** by **PCR** with primers flanking the *recA*::Kn ORF and for mitomycin-C sensitivity. Plasmid pKCT1-Tc, carrying the colicin K gene was constructed as follows. Firstly, a 1.8 kb blunt-ended fragment containing the Tc^r gene was obtained after digestion of vector pBR322 with restriction enzymes *Bsr*B I and *Bse*J I. Secondly, plasmid pKCT1 (Mulec *et al.*) was digested with *Psp1406* I to remove the β-lactamase gene, and the final fragment was treated to generate blunt ends (CloneJet kit; Fermentas). Both fragments were subsequently ligated and used to transform *E. coli* DH5α. Transformants were selected for tetracycline resistance.

Table 1: Bacterial strains and plasmid used in this study.
Tabela 1: Uporabljeni bakterijski sevi in plazmidi.

Strain	**Genotype**	**Source/Reference**
BW25113	*lacIq rrnBT14 DlacZWJ16 hsdR514 DaraBADAH33 DrhaBADLD78*	(Datsenko and Wanner 2000)
JW1889	BW25113 *araF*::Kn	Keio Collection
JW2669	BW25113 *recA*::Kn	Keio Collection
AB1157	*thr-1 leuB6 thi-1 supE44 lacY1 kdgK51 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 str-31 tsx-33*	(Bachmann 1987)
MG1655	*ilvG rfb-50 rph-1*	*E. coli* Genetic Stock Center (CGSC 6300)
RW118	*hr-1 araD139 D* (*gpt-proA*)*62 lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 xyl-5 mtl-1 argE3 thi-1 sulA211*	(Ho *et al*. 1993)
RW464	RW118 *recA1*	(Ho *et al*. 1993)
Plasmid		
pKCT1-Tc	pKCT1 with Ap^r gene replaced by Tc^r gene	(Mulec *et al*. 2003); this study

Determination of minimum inhibitory concentration (MIC)

LB medium was inoculated with a 1 percent inoculum of an overnight culture of *E. coli* and incubated at 37 °C until the culture reached a density of 0.5 McFarland's turbidity standard (optical density at 600 nm (OD_{600}) of approximately 0.1). A portion of the culture was diluted ten-fold with fresh LB medium, incubated for an additional 30 minutes at 37 °C and subsequently used at a 1:1000 dilution to inoculate test tubes with gradually increasing concentrations of antibiotics. Thus, the final cell number was approximately 5 x 10^4 per milliliter. After 18-24 hours of growth at 37 °C with aeration, the lowest concentration of antibiotic that has inhibited the visible growth of particular strain was determined.

Survival rate determination

An overnight culture was used to inoculate 40 ml of LB medium and incubated at 37 °C to an OD_{600} of 0.5. The culture was then divided into four 10 ml aliquots in shake flasks, one was used as a control, while appropriate concentrations of antibiotics were added to the others. Growth was continued with standard aeration at 37 °C. Samples were withdrawn at desired intervals and used for viable cell number determination. It was found that more accurate results of viable cell number were obtained when the antibiotic was removed from the sample prior to analysis by brief pelleting in a bench-top centrifuge. The sample was then resuspended in the original volume of fresh LB medium, followed by incubation of test tubes at 37 °C for 15 minutes in a water bath and then used for serial dilutions. This additional step greatly eliminated the presence of long filamentous, undivided cells as observed by microscopy (data not shown). All experiments were repeated at least three times. In addition, stock solutions of antibiotics were kept at -80 °C and used immediately after thawing and then discarded.

Colicin K induction

All $RecA^+$ strains were transformed with the colicin K producing plasmid pKCT1-Tc. Typically, an overnight culture grown at 37 °C was used to inoculate 40 ml of LB medium at a 1% inoculation level, which was incubated until an OD_{600} of 0.5 was reached. The culture was then divided into four 10 ml aliquots, one served as control, the second one was exposed to 0.5 μg/ml of mitomycin C (Sigma), the third to ampicillin at ¼ x MIC and the fourth to piperacillin at ¼ x MIC. Minimal inhibitory concentrations for particular strain are shown in Table 2. After growth for another 4 hrs, a 1 ml sample was removed. The cells were lysed by sonication (3 × 20 s bursts) using a Vibra Cell (Sonics) sonicator and a lysate was prepared by subsequent centrifugation at 20,000 x g for 10 min. Colicin K activity of the lysate was determined by agar diffusion on LB plates, seeded with *E. coli* DH5α as an indicator strain. A two-fold serial dilutions were used. It was found that the uninduced strain MG 1655 exhibited the lowest colicin K activity and thus, the highest dilution of its lysate still showing inhibition was defined as 1 arbitrary activity unit.

Results and Discussion

β-lactam survival rate of $RecA^-$/$RecA^+$ strains

Prior to comparing the survival of different *E. coli* mutants against β-lactam antibiotics, the minimum inhibitory concentrations (MIC) of the two antibiotics, ampicillin and piperacillin, was determined. Contrary to the results published by Miller et al. (2004), MICs for both antibiotics were substantially different, either lower or higher, in some commonly used isogenic $RecA^-$/$RecA^+$ strains (Tab. 2). To exclude the influence of possible secondary mutations, unintentionally introduced during preparation of *recA* defective strains, two fresh *recA* mutant strains, MG1644 *recA::Kn* and AB1157 *recA::Kn* were constructed. Introduction of a *recA* deletion mutation into strain MG1655, generally considered the least genetically manipulated *E. coli* strain, revealed that the observed MIC changes were indeed related to the *recA* mutation. As shown in Table 2, the MIC of ampicillin dropped from 7 μg/ml to 5 μg/ml and that of piperacillin from 2.5 μg/ml to 1.5 μg/ml. The observed strong sensitivity of all *recA* strains to the DNA damaging agent mitomycin C was as expected. However, MICs for unrelated targets (e.g.

Table 2: Growth characteristics of isogenic RecA$^-$/RecA$^+$ strains in presence of antibiotics. Final number of cells is expressed as mean ± standard deviation.
Tabela 2: Značilnosti rasti izogenih sevov RecA$^-$/RecA v prisotnosti antibiotikov. Končno število celic je izraženo kot povprečna vrednost ± standardna deviacija.

Parent strain	Mutant strains	MIC for ampicillin (μg/ml)	MIC for piperacillin (μg/ml)	MIC for chloramphenicol (μg/ml)	MIC for mitomycin (μg/ml)	Doubling time (min)	Final number of cells (x 10^9)
BW25113	w.t.	8.0	2.5	6.0	5.0	23	2.9 ± 0.6
	recA::Kn	5.0	1.5	3.5	0.2	40	0.3 ± 0.06
MG1655	w.t.	7.0	2.5	5.5	6.0	22	3.5 ± 0.9
	recA::Kn	5.0	1.5	4.0	0.4	29	2.1 ± 0.3
AB1157	w.t.	10.0	4.0	5.5	3.0	28	2.5 ± 0.2
	recA1	7.0	2.0	5.5	0.45	33	1.1 ± 0.2
	recA::Kn	7.0	2.5	5.5	0.4	37	1.2 ± 0.4
RW118	w.t.	9.0	2.5	3.5	6.0	25	2.6 ± 0.3
	recA1	7.0	2.0	3.0	0.25	40	0.75 ± 0.15

chloramphenicol) were also significantly reduced. In addition, some growth characteristics were also affected. All *recA* defective strains exhibited slower growth rates in comparison with the corresponding wild-type strains e.g., as much as twofold in the JW2669 (RecA$^-$) when compared with the isogenic strain JW1889 (RecA$^+$). Furthermore, the maximum cell concentration in a culture was also affected by mutation of *recA*, again most pronounced in strain JW2669 – the number of cells was reduced by as much as one order of magnitude. The striking difference between this particular strain and other *recA* mutants is not understood; none of the known mutations in JW1889, its parental strain, seem to be associated with any *recA* dependent process. Reduced viability of *recA* mutants was already observed by Miranda and Kuzminov (2003), although a direct comparison is not possible, as they cultivated their cultures under suboptimal conditions (at 28 °C).

Next, the survival rate of isogenic RecA$^-$/RecA$^+$ strains following addition of β-lactam antibiotics in the exponential growth phase was determined. The concentrations used were chosen in accordance with the obtained MIC data and at similar multiples (1/2, 1 and 2) of MICs to those used by Miller et al. (2004). The obtained results are presented in Table 3 for ampicillin and in Table 4 for piperacillin, respectively. As shown for both antibiotics, no significant difference in the survival rate of the isogenic RecA$^-$/RecA$^+$ strains was found. Clearly, our data did not confirm the previously reported assertion that inactivation of *recA* increases bacterial susceptibility to ampicillin and piperacillin.

SOS induction

Colicins, bacteriocins produced by *E. coli* strains, are typically regulated by the SOS response. To test the ability of either ampicillin or piperacillin to induce the SOS response, we studied the effect of both antibiotics on colicin K expression. Although the *cka* gene is transcribed-late during the SOS response, induction resulted in an increase of *cka* expression spanning three orders of magnitude (Tab. 5). Thus, even a slight change in *cka* gene activity could be easily identified. Nevertheless, our results (Tab. 5) showed no *cka* induction by ampicillin or piperacillin at ¼ x MIC. As expected, colicin K expression was fully induced by mitomycin C.

In conclusion, inactivation of *recA* results in important alterations in cell metabolism: (a) reduction of growth rate; (b) reduction of final cell density to only 10 % to 60 % of the wild-

Table 3: Survival of various isogenic RecA$^-$/RecA$^+$ strains following treatment with ampicillin. Percentage of survivors resembles the ratio of cell count before and and after the addition of antibiotic and is expressed as mean ± standard deviation.

Tabela 3: Preživetje različnih izogenih sevov RecA$^-$/RecA$^+$ po izpostavljenju ampicilinu. Odstotek preživetih je razmerje med številom celic pred in po dodatki antibiotika ter je izražen kot povprečna vrednost ± standardna deviacija.

Parent strain	Mutant strain	% of survivors					
		1/2 x MIC		1 x MIC		2 x MIC	
		1 h	4 h	1 h	4 h	1 h	4 h
BW25113	w.t.	32.8 ± 18.0	3.5 ± 1.0	10.2 ± 3.7	1.0 ± 0.7	4.1 ± 1.4	0.6 ± 0.3
	recA::Kn	80.1 ± 32.7	35.5 ± 1.5	8.1 ± 1.4	5.2 ± 2.3	1.7 ± 1.3	0.4 ± 0.3
MG1655	w.t.	61.6 ± 25.7	44.9 ± 27.8	22.8 ± 13.5	0.8 ± 0.6	1.8 ± 0.8	0.42 ± 0.39
	recA::Kn	75.9 ± 14.6	40.4 ± 22.4	32.7 ± 7.6	0.55 ± 0.28	2.1 ± 0.9	0.14 ± 0.03
AB1157	w.t.	105.7 ± 19.7	32.7 ± 15.0	51.1 ± 14.4	4.4 ± 1.9	20.6 ± 7.7	0.1 ± 0.06
	recA1	105.1 ± 27.5	17.0 ± 2.2	41.2 ± 8.1	1.9 ± 0.8	35.3 ± 4.2	0.6 ± 0.4
	recA::Kn	90.8 ± 11.6	65.1 ± 22.0	38.5 ± 19.0	7.9 ± 4.8	33.9 ± 18.6	3.3 ± 1.1
RW118	w.t.	48.8 ± 25.3	5.2 ± 2.9	17.1 ± 8.5	0.7 ± 0.56	1.5 ± 0.8	0.32 ± 0.2
	recA1	40.2 ± 10.3	12.6 ± 11.0	13.0 ± 3.7	0.9 ± 1.3	0.9 ± 0.6	0.08 ± 0.05

Table 4: Survival of various isogenic RecA$^-$/RecA$^+$ strains following treatment with piperacillin. Percentage of survivors resembles the ratio of cell count before and and after the addition of antibiotic and is expressed as mean ± standard deviation.

Tabela 4: Preživetje različnih izogenih sevov RecA$^-$/RecA$^+$ po izpostavljenju piperacilinu. Odstotek preživetih je razmerje med številom celic pred in po dodatki antibiotika ter je izražen kot povprečna vrednost ± standardna deviacija.

Parent strain	Mutant strain	% of survivors					
		1 x MIC		2 x MIC		4 x MIC	
		1 h	4 h	1 h	4 h	1 h	4 h
BW25113	w.t.	33.1 ± 12.1	12.4 ± 7.4	21.8 ± 12.9	6.5 ± 4.8	16.5 ± 8.2	1.4 ± 1.0
	recA::Kn	39.8 ± 12.3	33.9 ± 7.0	27.5 ± 15.7	19.9 ± 10.8	16.3 ± 3.3	10.7 ± 5.2
MG1655	w.t.	29.9 ± 11.9	8.4 ± 5.5	13.6 ± 4.9	6.9 ± 3.8	15.8 ± 6.1	4.9 ± 3.9
	recA::Kn	56.5 ± 10.9	3.4 ± 2.2	32.5 ± 7.9	3.3 ± 2.5	35.6 ± 8.0	2.8 ± 2.3
AB1157	w.t.	82.4 ± 16.5	7.5 ± 3.1	64.0 ± 19.5	9.0 ± 5.5	43.0 ± 21.6	4.2 ± 0.5
	recA1	68.0 ± 34.0	14.5 ± 4.5	52.2 ± 21.7	2.8 ± 1.3	23.5 ± 3.1	2.2 ± 1.6
	recA::Kn	59.1 ± 15.5	7.1 ± 2.2	37.2 ± 12.0	4.4 ± 1.3	27.2 ± 13.8	3.8 ± 1.4
RW118	w.t.	42.2 ± 7.1	7.5 ± 3.8	29.9 ± 11.9	2.1 ± 1.0	30.9 ± 8.4	2.5 ± 1.7
	recA1	34 ± 10.5	12.3 ± 5.3	24.9 ± 11.3	6.3 ± 3.8	19.3 ± 12.0	3.9 ± 1.6

Table 5: The activity of colicin K synthesized in *E.coli* RecA$^+$ strains of different genetic backgrounds following addition of ampicillin (1/4 of MIC), piperacillin (1/4 of MIC) or mitomycin C (0.5 µg/ml), respectively.
Tabela 5: Aktivnost kolicina K sintetiziranega v sevih *E. coli* RecA$^+$ različnega genetskega ozadja po dodatku bodisi ampicilina (1/4 MIC), piperacilina (1/4 MIC) ali mitomicina C (0.5 µg/ml).

Strain	Colicin K activity (arbitrary units)			
	no inducer/ control	ampicillin	piperacillin	mitomycin C
JW1889	2	2	1	1280-2560
MG1655	1	1	1-2	1280
AB1157	8	8	8	1280
RW118	8	8	4	2560-5120

type strain, depending on the genetic background (Tab. 2); and (c) increased sensitivity to different antibiotics with unrelated mode of action. In this respect, the interpretation of an isolated phenomenon, such as the response to β -lactams, could be misleading if based solely on the comparison between RecA$^-$/RecA$^+$ strains.

Summary

Due to the claim that β-lactam antibiotics induce the SOS response in *E. coli*, the characteristics of various *recA* mutants were analyzed. It was shown that inactivation of the *recA* gene severely altered cell metabolism, reflected in its growth rate, final population density and its sensitivity to unrelated antibiotics. By using genetically "clean" wild-type strains, it was possible to show that there is no link between the presence of β-lactam antibiotics and the SOS response.

The observations presented here urge the reinterpretation of the published results based on the genetically over-manipulated strains.

Povzetek

Zaradi trditve, da β-laktamski antibiotiki sprožijo odziv SOS pri *E. coli*, smo analizirali značilnosti nekaterih mutant *recA*. Pokazalo se je, da inaktivacija tega gena izzove dramatične spremembe v celičnem metabolizmu, ki se odražajo v stopnji rasti, končni populaciji celic in občutljivosti za različne, nesorodne antibiotike. Uporaba genetsko "čistega" seva je pokazala, da ni nobene povezave med β-laktamskimi antibiotiki in odzivom SOS.

Predstavljena opazovanja kličejo po nujni reinterpretaciji marsikaterega objavljenega rezultata, izvedenega na genetsko (pre)obremenjenih sevih.

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