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## Loss of topoisomerase I function affects the RpoS-dependent and GAD systems of acid resistance in *Escherichia coli*

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### SUMMARY

Acid resistance (AR) for *Escherichia coli* is important for its survival in the human gastrointestinal tract and involves three systems. The first AR system is dependent on the sigma factor RpoS. The second system (GAD system) requires glutamate decarboxylase isoforms encoded by the *gadA* and *gadB* genes. The third system (ARG system) requires arginine decarboxylase encoded by *adiA*. Loss of topoisomerase I function from *topA* deletion or Tn10 insertion mutations lowered the resistance to killing by pH 2 or 2.5 treatment by 10 to >100 fold. The RpoS and GAD systems were both affected by the *topA* mutation but the ARG system of acid resistance was not affected. Northern blot analysis showed that induction of *gadA* and *gadB* transcription in stationary phase and at pH 5.5 was decreased in the *topA* mutant. Western blot analysis showed that the *topA* mutation did not affect accumulation of RpoS, GadX or GadW proteins. Topoisomerase I could have a direct influence on transcription of acid resistance genes. This influence did not involve R-loop formation as the overexpression of RNase H did not alleviate the decrease of acid resistance from the *topA* mutation. The effect of the *topA* mutation could be suppressed by the *hns* mutation so topoisomerase I might be required to counteract the effect of H-NS protein on gene expression in addition to its influence on RpoS-dependent transcription.

### INTRODUCTION

For pathogenic or non-pathogenic *Escherichia coli* to colonize human gastrointestinal tracts, it has to survive the challenge of extreme acid pH (Giannella *et al.*, 1972; Gordon & Small, 1973). Enterohemorrhagic *E. coli* strains require a low infectious dose (ID) to cause disease due partly to their high degree of acid resistance (AR) to pH of 2.5 or lower (Benjamin & Datta, 1995; Conner & Kotrola, 1995; Lin *et al.*, 1996, Price *et al.*, 2004). Three distinct acid resistance systems are responsible for protecting stationary phase *E. coli* cells from killing

by the extreme low pH (Castanie-Cornet *et al.*, 1999; Foster, 2004). The oxidative or glucose-repressed system (System 1) is dependent on the stationary-phase sigma factor RpoS (Small *et al.*, 1994). The GAD system requires glutamate during acid challenge and involves glutamate decarboxylase activity (Lin *et al.*, 1995; Hersh *et al.*, 1996; De Biase *et al.*, 1999). The ARG system requires arginine in the acid medium and arginine decarboxylase activity (Lin *et al.*, 1995; Castanie-Cornet *et al.*, 1999). RpoS is also required for expression of *gadA* and *gadBC*, the structural genes for glutamate decarboxylase isozymes, at neutral pH in the stationary phase (Castanie-Cornet *et al.*, 1999; Castanie-Cornet & Foster, 2001).

Topoisomerase I is the major activity for removal of negative supercoils from DNA in *E. coli*, and along with DNA gyrase and topoisomerase IV, plays an important role in regulation of both global and localized supercoiling (Zechiedrich *et al.*, 2000; Champoux, 2001). Four promoters have been characterized for the *E. coli topA* gene encoding topoisomerase I (Qi *et al.*, 1997). Promoter P1 is recognized by the heat shock responsive sigma factor  $\sigma^{32}$  (Lesley *et al.*, 1990; Qi *et al.*, 1996) while promoter Px1 can be recognized by the stationary phase and stress responsive sigma factor  $\sigma^s$  (RpoS). The transcription of *topA* by these sigma factors in response to environmental challenge suggests a role of topoisomerase I function in adaptation and survival of *E. coli*. This hypothesis is supported by the lower survival rates of the *topA* deletion mutants versus *topA*<sup>+</sup> isogenic strains after challenges with high temperature or oxidative stress (Qi *et al.*, 1999; Tse-Dinh, 2000). Loss of topoisomerase I activity can affect transcription initiation at promoters due to its influence on DNA topology (Steck *et al.*, 1993; Wang & Lynch, 1993), and is needed during transcription elongation to suppress hypernegative supercoiling and R-loop formation (Massé & Drolet, 1999; Hraiky *et al.*, 2000). Efficient transcription of genes required for acid resistance may thus require topoisomerase I activity to be present in the cell. In *Helicobacter pylori*, *topA* was found by differential display PCR to be one of the genes induced by prolonged acid exposure (Dong *et al.*, 2001), suggesting the involvement of topoisomerase I in adaptation to low pH by this organism. We now present results of experiments that demonstrate the effect of *topA* mutation on *E. coli* acid resistance, and on the expression of genes involved in the oxidative and GAD acid resistance mechanisms.

## METHODS

### Bacterial strains, plasmids and culture media

The bacterial strains and plasmids used in this study are described in Table 1. P1 transduction was carried out according to standard protocols (Miller, 1992). The insertion mutation in *rpoS* or *topA* mutant strains selected by Tet<sup>r</sup> was confirmed by Western blotting with antibodies against the targeted protein. *E. coli* was grown at 37°C in Luria Broth (LB), LB supplemented with 0.4% glucose (LBG), or LB buffered with either 100 mM morpholinepropanesulfonic acid (MES; pH 5.5) or with 100 mM morpholinepropanesulfonic acid (MOPS; pH 8). Overall acid resistance (AR) is measured by diluting the culture into LB pH 2.0 or pH 2.5 after overnight growth in LB buffered at pH 5.5 or pH 8.0 (Castanie-Cornet *et al.*, 1999). To test the individual acid resistance system, survival rates were measured after treatment in E minimal medium (Vogel & Bonner, 1956) containing 0.4% glucose (EG) at pH 2.5 after different overnight growth conditions as

described (Castanie-Cornet *et al.*, 1999). For System 1 (oxidative system), overnight growth was in LB pH 5.5, followed by treatment in EG pH 2.5 with no supplement. For the GAD acid resistance system, overnight culture in LBG was diluted into EG pH 2.5 supplemented with 1.5 mM glutamate. For the ARG acid resistance system, overnight growth was in brain heart infusion medium supplemented with glucose (BHIG), followed by treatment in EG pH 2.5 supplemented with 0.6 mM arginine. In the experiments involving *hns* mutants, M9 medium at pH 2.5 with 0.4% glucose was used as the acid challenge medium (Hommals *et al.*, 2001). After challenge for the indicated period of time, the viable counts were determined by dilutions in M9 minimal medium (Sambrook *et al.*, 1989) and plating on LB plates, supplemented by ampicillin (100  $\mu\text{g ml}^{-1}$ ), tetracycline (15  $\mu\text{g ml}^{-1}$ ), or kanamycin (25  $\mu\text{g ml}^{-1}$ ) when appropriate.

### Northern blotting and real-time PCR

Total RNA was isolated from cells grown in buffered LB to OD<sub>600</sub> 0.4–0.5 (EP, exponential phase) or 1.2–1.5 (SP, early stationary phase) using previously published procedures (Hraiky *et al.*, 2000). The 1.4 kb *gad* probe for detection of both *gadA* and *gadBC* was generated by PCR as described previously (Castanie-Cornet & Foster, 2001) and labeled with [ $\alpha$ -<sup>32</sup>P]dCTP using the Mega Prime kit from Amersham. Electrophoresis and transfer of RNA samples (5  $\mu\text{g}$ ) were carried out as described (Sambrook *et al.*, 1989). The PerfectHyb Plus solution (from Sigma) was used for hybridization. For real-time PCR analysis, RNA was treated with DNase I (Promega) prior to cDNA synthesis with random primers and AMV reverse transcriptase (Promega). Primers 5'-GAACTGGCCACTTTCTGC-3' and 5'-GCGCATAGGGATCTCACG-3' were used to amplify a 376 bp fragment from both *gadA* and *gadB* cDNA using the Roche Life-Cycler.

### Western blotting

Cells from exponential phase or overnight cultures were collected by centrifugation and lysed by boiling in the sample buffer for SDS gel electrophoresis for 5 min. The total lysates with volumes adjusted for cell densities were electrophoresed in 10% SDS polyacrylamide gels. The proteins in the gel were either stained with coomassie blue to check for equal loading or transferred onto nitrocellulose filters for Western blotting using the ECL Plus reagents (Amersham). The antibodies against *E. coli* topoisomerase I (Qi *et al.*, 1996) and GAD (Castanie-Cornet *et al.*, 1999) were described previously. Mouse monoclonal antibodies against *E. coli* RpoS was purchased from Neoclone. Antibodies against GadW were raised in rat using the peptide sequence YHEQQKISLHNESILC. Antibodies against GadX (Shin *et al.*, 2001) were kindly provided by Dr. J. B. Kaper. The coomassie stained gels and exposed films were analyzed by the Alpha-Imager for quantitation.

## RESULTS

### Loss of topoisomerase I function leads to reduced acid resistance

The increased sensitivity of the *topA* deletion mutant strain RFM475 to high temperature and oxidative stress challenge when compared to its isogenic *topA*<sup>+</sup> counterpart, RFM445, has been reported (Qi *et al.*, 1999; Tse-Dinh, 2000). The effect of loss of topoisomerase I function on acid resistance was investigated here. After overnight growth in either LB

buffered at pH 8.0 or 5.5, RFM475 was found to have 20–30 fold lower acid resistance than RFM445 (Fig. 1). The *cysB* gene function, also missing in RFM475 due to the *topA-cysB* deletion, is required for the induction of the arginine decarboxylase activity in the ARG acid resistance mechanism (Shi & Bennett, 1994; Lin *et al.*, 1996). This loss of *cysB* function is expected to lead to decreased arginine decarboxylase-dependent acid resistance in RFM475. We therefore also tested a *topA20::Tn10* insertion mutant (YT481) that is wild-type for *cysB* function for acid resistance and found similar degree of sensitivity due to the *topA* mutation in the absence of the *cysB* mutation (Fig. 1). This indicates that *topA* mutations affects acid resistance in mechanisms independent of the arginine decarboxylase activity. Topoisomerase I protein was not detected in the total protein of YT481 by Western blot analysis (data not shown).

### Loss of topoisomerase I function affects the oxidative and GAD systems of acid resistance

To demonstrate directly which of the three acid resistance mechanisms was affected by the loss of topoisomerase I function, the three AR systems were tested individually and the results are shown in Fig. 2. As expected from the loss of the *cysB* function, RFM475 was defective in arginine-dependent AR. However, the arginine-dependent AR was not affected in YT481 with the *topA20::Tn10* mutation, indicating that topoisomerase I function is not required for the arginine-dependent acid resistance mechanism. The oxidative system and glutamate-dependent systems were both affected by the loss of topoisomerase I function in the two *topA* mutants. However, the two *topA* mutants differed in the relative degree of sensitivity. Mutant RFM475 appeared to be more severely affected in the oxidative system while the glutamate-dependent system was more severely affected in mutant YT481. The partial loss of oxidative AR in mutant YT481 was confirmed by longer exposure to the pH 2.5 EG medium. After 6 h of exposure, the survival rate of YT481 was 0.046% versus 7.1% for RFM445.

### Overexpression of RNase H does not improve the AR of *topA* mutants

In *topA* mutants, the formation of R-loops during transcription elongation is driven by the accumulation of hypernegative supercoiling. The blocking of RNA polymerase by these R-loops can account at least partially the growth defect of *topA* mutants (Drolet *et al.*, 1995; Massé & Drolet, 1999; Baakli *et al.*, 2004). Overexpression of RNase H in *topA* mutants including RFM475 has been shown to correct this defect of R-loop formation (Drolet *et al.*, 1995; Hraikly *et al.*, 2000). The overexpression of RNase H from plasmid pSK760 has also been shown to partially alleviate the effect of *topA* deletion on the survival rate of RFM475 after high temperature and oxidative challenge (Cheng *et al.*, 2003). We therefore compared the survival rates of *topA* mutants transformed with either pSK760 expressing wild type RNase H or pSK762c expressing inactive RNase H mutant. The results (Table 2) showed that there was no significant increase in the overall AR (left column) or GAD-dependent AR (right column) of RFM475 or YT481 that could be attributed to the overexpression of RNase H when the pSK760 plasmid was present.

### Effect of Topoisomerase I function on the level of glutamate decarboxylase isoforms

The glutamate decarboxylase activity encoded by *gadA* and *gadB* consumes an intracellular proton for the decarboxylation of each molecule of glutamate. It thus plays a major role in

acid resistance. Transcription of both *gadA* and *gadBC* is positively controlled by the transcriptional activator GadX (Shin et al., 2001; Tramonti et al., 2002). RpoS is also involved in the induction of *gad* and *gadB* during the stationary phase of cell growth (Castanie-Cornet & Foster, 2001). The total level of GadA and GadB proteins in RFM445 and the *topA* mutants RFM475, YT481 was measured by Western blotting. The results (Fig. 3) showed that the GadA and GadB proteins were present in significantly lower level in the *topA* mutants after overnight growth in both neutral (pH 8) and acidic pH (pH 5.5). The decrease of GadA/B resulting from the *topA* mutation was also seen under conditions with active oxidative system (during stationary phase in LB or EG pH 5.5 medium) or repressed RpoS activity (during exponential phase in EG medium or during stationary phase in LBG medium). This decrease in GadA and GadB proteins in the *topA* mutants would account for the diminished acid resistance observed under these different growth conditions.

### **TopA mutation diminished the induction of *gadA* and *gadBC* genes in acidic LB during stationary phase**

In order to determine if transcription of *gadA* and *gadBC* genes was affected by the *topA* mutation, total RNA was prepared and used for Northern blotting. The results (Fig. 4) showed that the induction of the *gadA* and *gadBC* message level during entry into stationary phase ( $OD_{600} = 1.4$ ) at acidic pH was drastically decreased in the *topA* mutants RFM475 and YT481 versus RFM445. The difference in *gadA* and *gadB* message levels in pH5.5 stationary phase LB cultures was quantified by real-time PCR. The ratio measured for YT481 versus RFM445 was found to be  $1:60 \pm 10$  (average of results from two independent RNA preparations in three separate experiments).

### **The levels of RpoS, GadX and GadW were not altered by the *topA* mutation**

The sigma factor RpoS is central to the resistance to acidic pH in the glucose-repressed mechanism in complex medium. The AraC-like regulators GadX and GadW have also been implicated in the complex regulation of glutamate decarboxylase system (Shin et al., 2001; Tramonti et al., 2002; Ma et al., 2002, 2003). Western blotting was used to compare the amounts of RpoS, GadX and GadW in the *topA*<sup>+</sup> and *topA* mutant cultures to determine if change in levels of these regulators accounted for the decreased *gadA* and *gadBC* expression in the *topA* mutants. The results showed that the levels of RpoS accumulated in overnight stationary phase cultures of the *topA* mutants RFM475 and YT481 were similar to those found in RFM445 (Fig. 5). There was also no difference observed for the level of GadX and GadW due to the *topA* mutation under different growth conditions (Fig. 5). Therefore topoisomerase I function had no significant effect on the accumulated levels of these regulators.

### **RpoS-independent mechanism also accounts for some of the effect of *topA* mutation**

A RpoS dependent promoter is involved in the transcription of *topA* (Qi et al., 1997), suggesting that topoisomerase I function may be important for gene expression directed by RpoS. The *rpoS*:Tn10 mutation was introduced into the strains RFM445 and RFM475 to determine if the effect of *topA* mutation on acid resistance was entirely dependent on transcription directed by RpoS. Survival in the absence of glutamate at pH 2.5 for 1 hour was 3.5% for YT445R (*rpoS*) and 0.098% for YT475R (*rpoS topA*). In the presence of

glutamate at pH 2.5 for 2 hours, survival was 0.1% vs 0.0029%, respectively. These results showed that the presence of *topA* mutation in the *rpoS* mutant background further decreased the survival rate. Therefore the effect of the *topA* mutation on acid resistance was not entirely due to RpoS-dependent transcription. Measurement of the level of GadA and GadB proteins by Western blot also showed that there was a further decrease of expression from the *topA* mutation in the *rpoS* null background (Fig 6a).

### The effect of *topA* deletion can be suppressed by *hns* mutation

H-NS protein is a negative regulator of acid resistance (Foster, 2004). The *hns* mutation affecting the nucleoid protein H-NS has been shown to increase expression of the *gadA* and *gadBC* genes (Yoshia et al., 1993a,b; De Biase et al., 1999; Hommals et al., 2001; Tramonti et al., 2002; Ma et al., 2002; Waterman & Small, 2003). The *hns*:Tn5 mutation was introduced into RFM445 and RFM475. Acid resistance of the resulting strains YT445H and YT475H were compared after using conditions previously used to demonstrate the increase in acid resistance from *hns* mutation (Hommals et al., 2001). The effect of the *topA* mutation in RFM475 versus RFM445 under these experimental conditions was >100 fold reduction in acid resistance (Table 3). There was a modest increase in acid resistance when the *hns* mutation was introduced into RFM445. The increase in acid resistance from the *hns* mutation was much greater in RFM475, restoring it close to the level observed for RFM445. The increase in acid resistance in the *hns* mutants correlates to the increase in expression of GadA and Gad B proteins as analyzed by Western blots (Fig. 6b).

### Effect of *gyrB* mutations and gyrase inhibition on acid resistance

The *topA*<sup>+</sup> strain RFM445 contains gyrase mutations necessary for survival of RFM475 and YT481. The acid resistance of the *gyr*<sup>+</sup> strain RFM443 was also measured to determine if the *gyrB221*(cou<sup>R</sup>), *gyrB203*(Ts) mutations in RFM445 had any effect. Overnight growth of RFM443 was also carried out in the presence of sub-lethal concentrations of novobiocin to determine if inhibition of gyrase activity influenced acid resistance. Besides the conditions of adaptation in pH 5.5 M9 medium used previously to test the effect of the *hns* mutation (Hommals et al., 2001), the RpoS suppressed condition (overnight growth in LBG) with the relatively lower survival rates for the *topA*<sup>+</sup> strains was also tested, so that any positive effect from the reduced gyrase activity could potentially be detected. The results (Table 4) showed that the *gyrB* mutations in RFM445 resulted in a small increase (~2 fold) of the RpoS-independent and glutamate-dependent acid resistance after overnight growth in LBG. This could be due to the moderate level of reduction of gyrase activity in RFM445, compared to topoisomerase I activity missing totally in the *topA* mutants. However, the gyrase mutations had the opposite effect when acid resistance was measured after overnight growth in M9 medium at pH 5.5. The presence of novobiocin at 15 and 30 µg/ml inhibited overnight growth of RFM443 by about ten fold (data not shown). There was a small decrease in acid resistance after adaptation in LBG (~2–3 fold) and a more significant decrease in acid resistance after adaptation in pH5.5 M9 medium (up to ten fold).

## DISCUSSION

Glutamate decarboxylase activity plays an important role in the acid resistance of *E. coli*. Data presented here showed that a *topA* mutation resulted in significantly lower transcription of *gadA* and *gadBC*, accounting at least in part for the decreased acid resistance of the *topA* mutants. Unlike other growth defects of *topA* mutants including increased sensitivity to high temperature and oxidative stress, the reduced acid resistance could not be rescued by the overexpression of RNase H. Therefore it is unlikely for R-loop accumulation during transcription elongation to be the cause of the reduced acid resistance even though it could conceivably be argued that degradation of the mRNA required for acid resistance by the overexpressed RNase H led to the low level of survival.

Expression of *gadA* and *gadBC* in stationary phase has been shown to be largely dependent on RpoS (Castanie-Cornet et al., 1999; De Biase et al., 1999). This is mediated by the RpoS-dependent transcription of GadX, an activator of *gadA* and *gadBC* (Ma et al., 2000). Our results showed that the decreased survival of the *topA* mutants under the RpoS-dependent mechanism of acid resistance was not due to a reduction in level of RpoS or GadX protein. The loss of topoisomerase I function could have affected the transcription of other RpoS target promoters directly, since high level of negative supercoiling has been reported to inhibit transcription by RpoS (Bordes et al., 2003), and localized hypernegative supercoiling might be present in the *topA* mutants. It was found that the gyrase inhibitor novobiocin induced the RpoS-directed transcription initiation from the *osmE<sub>p</sub>* gene without affecting the level of RpoS accumulation (Bordes et al., 2003). However, in this study the introduction of gyrase mutations or the addition of novobiocin to RFM443 resulted in only small changes in acid resistance, and not always in the opposite direction of *topA* mutation. In addition, the data from the *rpoS* mutants indicated that topoisomerase I function was also involved in an RpoS-independent mechanism of acid resistance.

The nucleoid protein H-NS has been shown to act as a negative regulator on *E. coli* genes required for acid resistance (Yoshida et al., 1993a,b; Hommala et al., 2001). The repression of gene expression by H-NS may be due to its effect on DNA supercoiling (Tupper et al., 1994) or its binding to specific curved DNA sites in the promoter regions of these genes. The large effect of the *hns* mutation on strain RFM475 relative to RFM445 suggested that topoisomerase I function may be required to overcome the repression of acid resistance genes by H-NS. It has been shown that H-NS can repress *gadA* and *gadBC* expression independent of GadX (Ma et al., 2002). The effect of *hns* mutation on *gadA* and *gadBC* transcription remained highly significant even in a *rpoS* mutant background (Waterman & Small, 2003), so this might account for the observation that the *topA* effect could still be seen in the *rpoS* mutants studied here. Topoisomerase I function may be needed to counteract the H-NS repression on *gadA* and *gadBC* expression. The role of topoisomerase I at such loci is not simply due to its relaxation activity, since the addition of novobiocin resulted in decrease of acid resistance.

A previous study with *Salmonella typhimurium* established that DNA topology can affect the environmental regulation of the pH-regulated locus *aniG* (Karem & Foster, 1993). Expression of *aniG* in mannose media is decreased in a *topA* mutant, but increased in two

*hns* mutants at pH 6.0–7.0 (Karem & Foster, 1993). This is also in agreement with a possible role of topoisomerase I in relieving repression of *aniG* by H-NS. Many other bacterial genes including some of the genes involved in virulence and osmotic control are also known to be regulated by H-NS (Hommais et al., 2001; Madrid et al., 2002). The *virF* gene of *Shigella* required for invasion functions is suppressed by interaction of H-NS with its promoter, and its thermal induction was found to be inhibited in a *topA* mutant (Ni Bhriain & Dorman, 1993). This is also similar to the effect of *topA* on the induction *gad* expression during stationary phase observed here. The effective DNA superhelicity at any gene promoter region is a result of the local dynamic competition between structural constraining proteins, including H-NS, RNA polymerase and the topoisomerases (Travers & Muskhelishvili, 2005). This dynamic competition changes during growth-phase transition and adaptive response to challenge. Topoisomerase I function could have a direct role in relieving repression of H-NS at specific gene loci, but it also cannot be ruled out that topoisomerase I may be counteracting the general effect of H-NS on DNA topology in the other cases (Mojica & Higgins, 1997). In a study of *E. coli* protein-protein interaction network, topoisomerase I was found to interact H-NS (Butland *et al.*, 2005), suggesting a functional link between the two proteins. We have demonstrated previously that *E. coli* topoisomerase I function was required for R-loop suppression during transcription elongation of stress genes during adaptation to high temperature and oxidative stress (Cheng et al., 2003). In this study, it was found that *E. coli* topoisomerase I played a different role in gene expression for acid resistance that could involve the influence of DNA topology on the action of the positive and negative regulators of transcription. This further supports the importance of type IA topoisomerase function during environmental adaptation and its conservation in evolution (Wang, 2002). Furthermore inhibitors of bacterial topoisomerase I activity may be useful for general suppression of adaptation of pathogenic bacteria to host environments.

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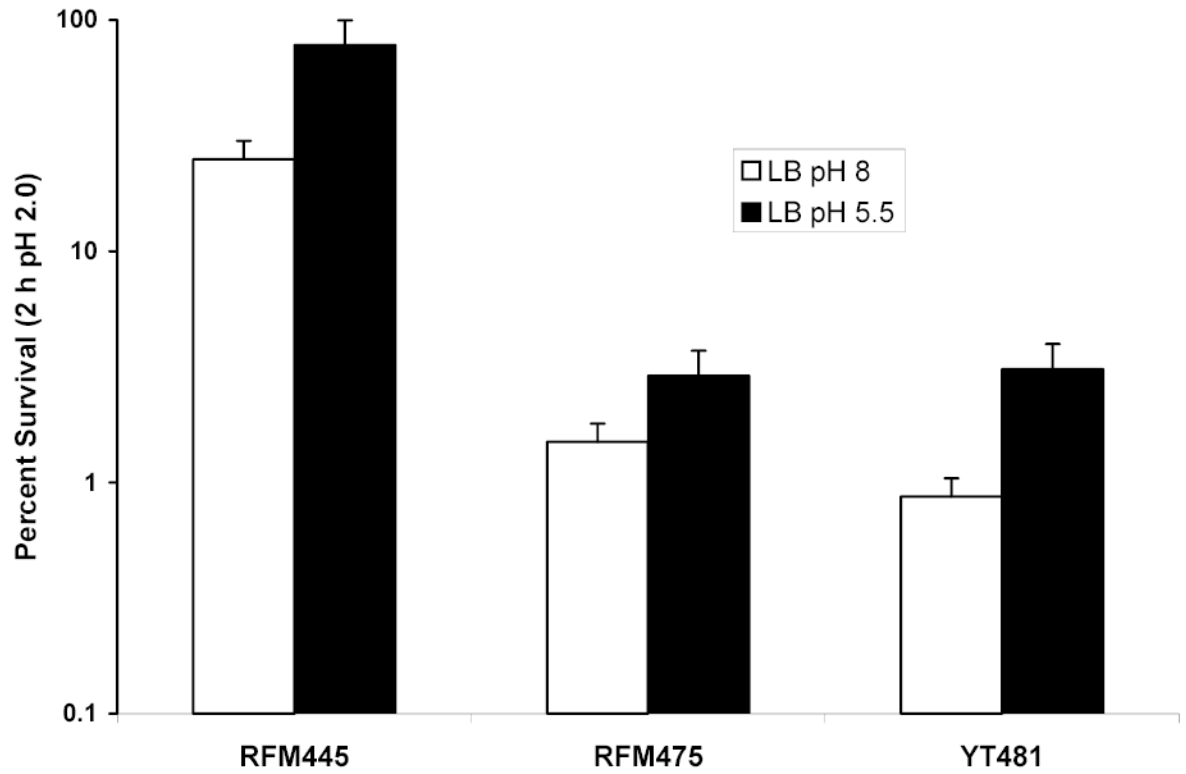
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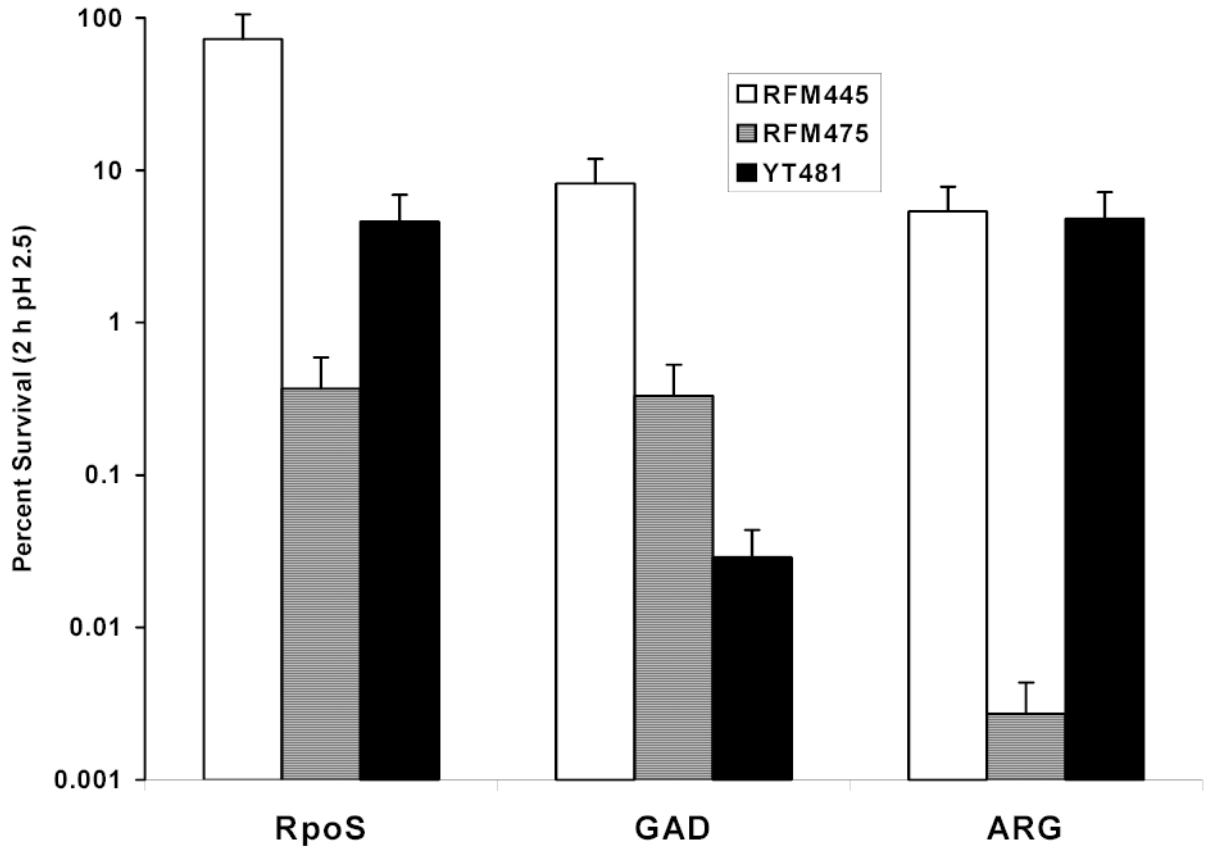
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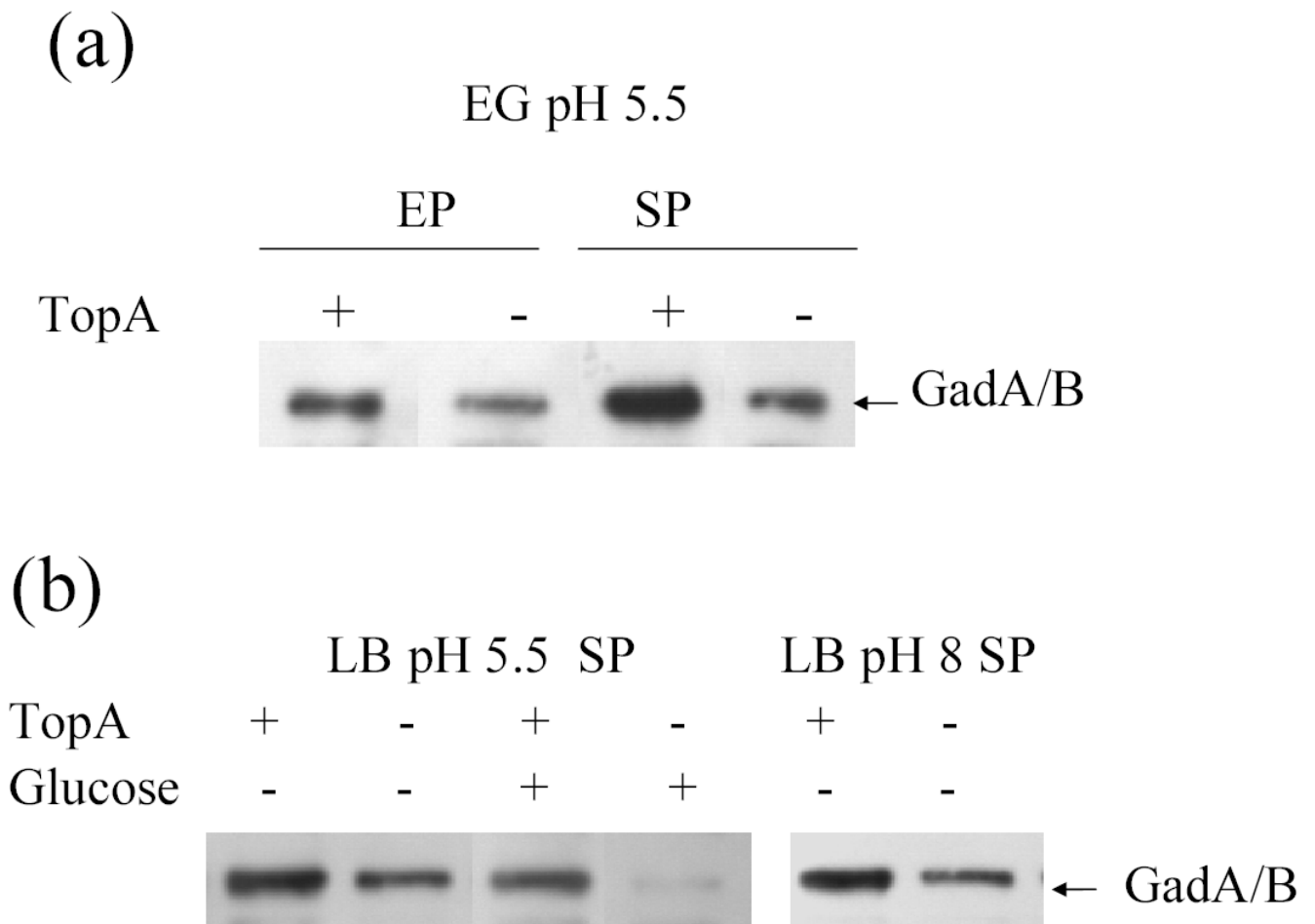


**Fig. 1.** Effect of *topA* mutation on overall acid resistance as measured by survival rates after overnight growth in LB pH 5.5 or 8.0, and challenge in LB pH 2.0 for 2 h. The results represent the average from three independent experiments.

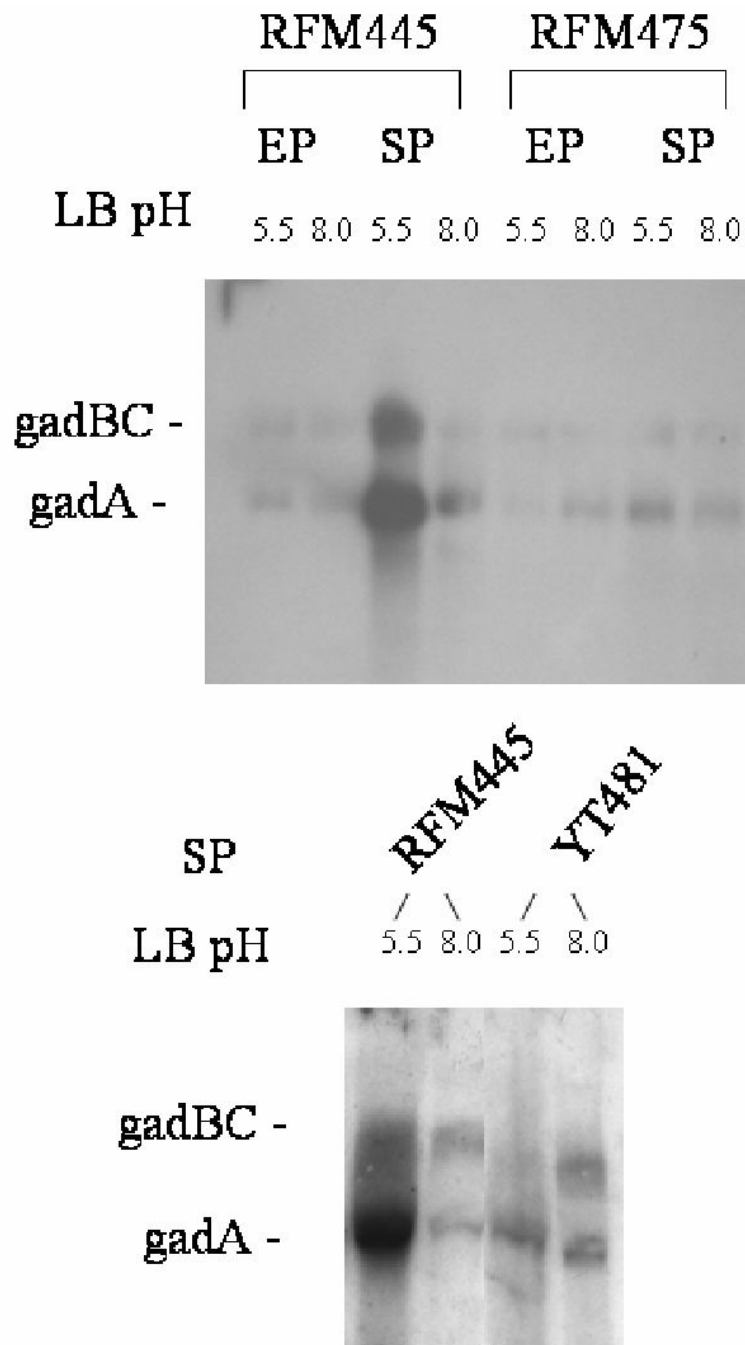


**Fig. 2.**

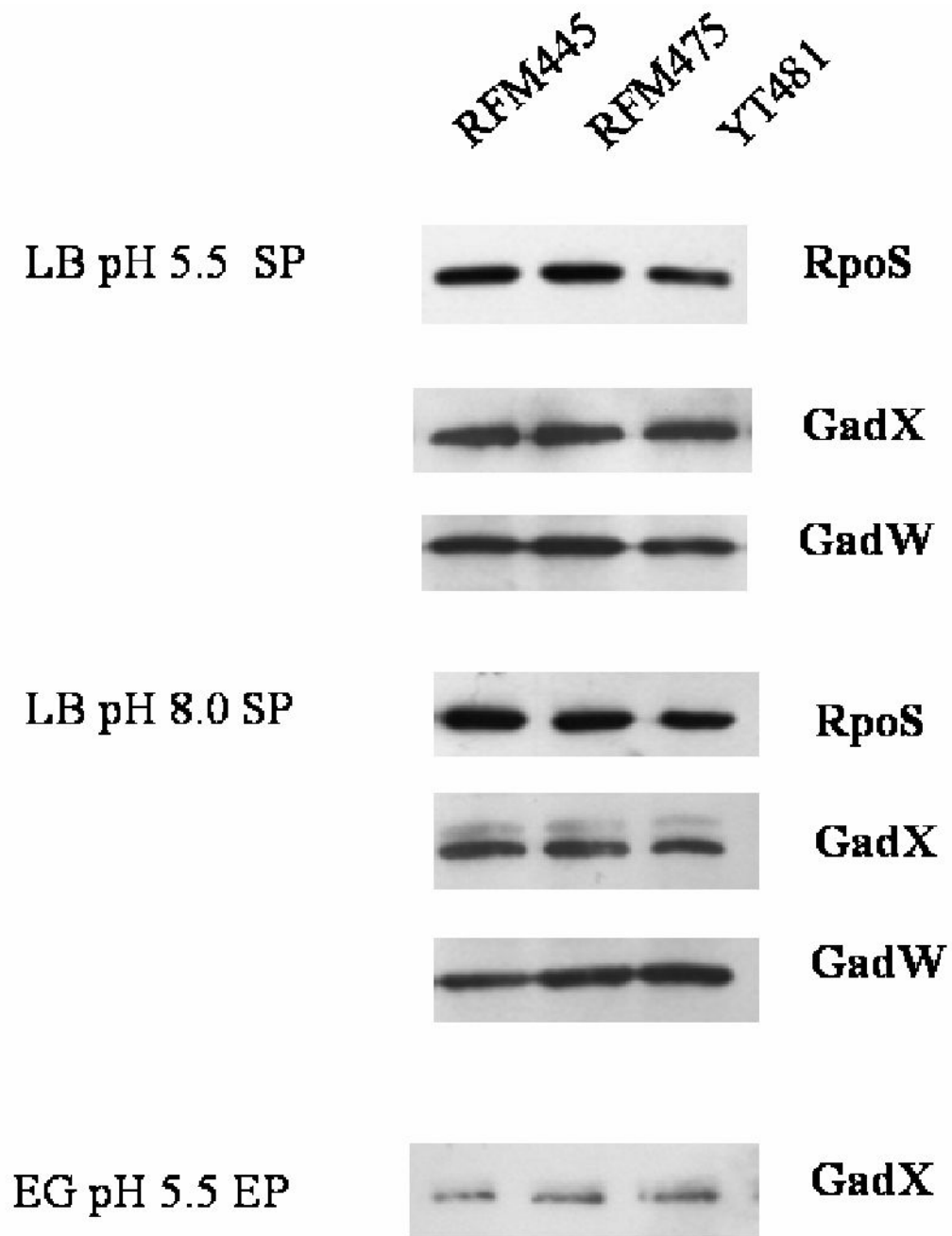
Effect if *topA* mutation on the three different mechanisms of acid resistance. The test for the oxidative system (RpoS) involved overnight growth in LB-MES (pH5.5) followed by dilution into EG (pH 2.5). Survival (viable counts) was measured after 2 h at 37°C. The glutamate (GAD) and arginine (ARG) systems required overnight LBG (glutamate system) or BHIG (arginine system) cultures which were diluted into EG (pH 2.5) containing 1.5 mM glutamate or 0.6 mM arginine respectively. The deletion of *cysB* gene in RFM475 ( *topAcysB*) accounts for the low acid resistance from the arginine-dependent mechanism. Strain YT481 (*topA20::Tn10*) has wild-type *cysB* gene. The results represent the average from four independent experiments.



**Fig. 3.** Effect of *topA* mutation on the GadA and GadB protein levels determined by western blot analysis. (a) RFM445 (*topA*<sup>+</sup>) and YT481(*topA20::Tn10*) were grown in EG pH5.5 medium to EP (OD<sub>600</sub>=0.5) and SP (18 h). (b) RFM445 and RFM475 (*topA*<sup>-</sup>) were grown in buffered LB for 18 h with either no glucose or 0.4% glucose added.

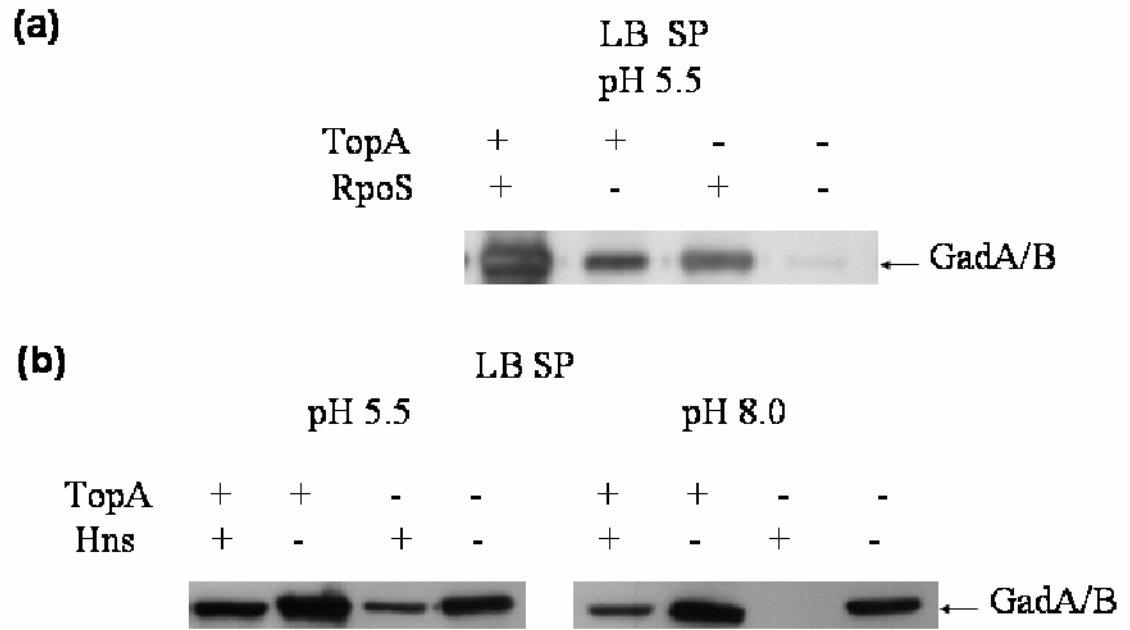


**Fig. 4.** Effect of *topA* mutation on the *gadA* and *gadBC* mRNA levels determined by Northern blot analysis. RNA was extracted from RFM445 (*topA*<sup>+</sup>), RFM475 (*topA*<sup>-</sup>) and YT481 (*topA20::Tn10*) grown in LB at pH 5.5 or pH 8.0 to exponential phase (EP) or early stationary phase (SP).



**Fig. 5.** Western blot analysis to compare levels of RpoS, GadX and GadW proteins in RFM445 and its *topA* mutant derivatives.





**Fig. 6.**

Western blot analysis of the effect of *topA* mutation on the GadA and GadB protein levels in the presence of background mutation of either (a) *rpoS* (b) *hns*. Mutations of *rpoS* or *hns* were introduced by transduction into RFM445 (*topA*<sup>+</sup>) and RFM475 (*topA*<sup>-</sup>)

**Table 1***E. coli* strains and plasmids used in this study.

Strain or plasmid	Relevant genotype and characteristics	Reference or Source
<b><i>E. coli</i> strains</b>		
RFM443	<i>rpsL galK2 lac74</i>	Drolet <i>et al.</i> , 1995
RFM445	<i>rpsL galK2 lac74, gyrB221(cou<sup>R</sup>), gyrB203(Ts)</i>	Drolet <i>et al.</i> , 1995
RFM475	<i>rpsL galK2 lac74, gyrB221(cou<sup>R</sup>), gyrB203(Ts) (topAcysB)204</i>	Drolet <i>et al.</i> , 1995
2111	W3110, <i>gyrB(Ts, cou<sup>R</sup>), topA20::Tn10</i>	Zechiedrich <i>et al.</i> , 2000
YT481	RFM445 <i>topA20::Tn10</i>	P1(2111)x(RFM445), tet <sup>r</sup>
GY12	<i>rpoS::Tn10</i>	Yamashino <i>et al.</i> , 1995
YT445R	RFM445 <i>rpoS::Tn10</i>	P1(GY12)x(RFM445), tet <sup>r</sup>
YT475R	RFM475 <i>rpoS::Tn10</i>	P1(GY12)x(RFM475), tet <sup>r</sup>
FB20271	MG1655 <i>hns::Tn5</i>	F. Blattner Laboratory
YT445H	RFM445 <i>hns::Tn5</i>	P1(FB20271)x(RFM445), kan <sup>r</sup>
YT475H	RFM475 <i>hns::Tn5</i>	P1(FB20271)x(RFM475), kan <sup>r</sup>
<b>Plasmids</b>		
pSK760	pBR322 derivative with the <i>mhA</i> gene expressing RNase H	Kanaya & Crouch, 1983
pSK762c	pBR322 derivative with mutated <i>mhA</i> gene expressing inactive RNase H	Drolet <i>et al.</i> , 1995

**Table 2**  
**Effect of RNase overexpression from plasmid pSK760 on acid resistance of *topA* mutants**

Each experiment was carried out four times. Mean results and standard deviations are shown.

Overall Acid Resistance		GAD Acid Resistance	
Adaptation: Overnight in LB pH 5.5		Adaptation: Overnight in LBG	
Acid challenge: EG pH 2.5, 2 h		Acid challenge: EG pH 2.5 + Glutamate, 2 h	
Strain	Percent Survival	Strain	Percent Survival
RFM445	73±14	RFM445	8.2±3.2
RFM475 ( <i>topAcysB</i> )	0.37±0.18	YT481 <i>topA20::Tn10</i>	0.029±0.011
RFM475/pSK760	0.15±0.03	YT481/pSK760	0.030±0.012
RFM475/pSK762c	0.66±0.30	YT481/pSK762c	0.043±0.014

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**Table 3**  
***hns* mutation can suppress the effect of the *topA* mutation on acid resistance**

Each experiment was carried out four times. Mean results and standard deviations are shown.

<b>Adaptation: Overnight in M9 pH 5.5</b>	
<b>Acid challenge: M9 pH 2.5 + Glutamate, 2 h</b>	
<b>Strain</b>	<b>Percent Survival</b>
RFM445	47 ±9
YT445H <i>hns</i> ::Tn5	59 ±9
RFM475	0.25±0.09
YT475H <i>hns</i> ::Tn5	44 ±5

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**Table 4**  
**Effect of the *gyrB* mutations in RFM445 and inhibition of gyrase activity in RFM443 by novobiocin on acid resistance**

Each experiment was carried out four times. Mean results and standard deviations are shown.

Percent Survival			
RFM445 <i>gyrB221(cou<sup>R</sup>), gyrB203(Ts)</i>	RFM443	RFM443 15 µg/ml novobiocin	RFM443 30 µg/ml novobiocin
Overnight in M9 pH5.5, challenged for 2 hr in M9 pH 2.5 + glutamate			
47±9	90±10	25±6	8.0 ±1.0
Overnight in LBG, challenged for 2 hr in EG pH 2.5 + glutamate			
8.2±3.2	4.4±2.2	2.6±1.4	1.4±0.6

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