Activation of a Partial Stringent Response Protects Amino Acid-Deprived Escherichia coli from Ampicillin-Induced Lysis

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In amino-acid deprived Escherichia coli (E. coli), the activation of the stringent response by RelA and its key alarmone product, guanosine 3',5'-bispyrophosphate (ppGpp), is necessary for a variety of cellular responses, including tolerance to β-lactam antibiotics such as ampicillin. Ampicillin uncouples the activity of the peptidoglycan polymerases (penicillin-binding proteins) from the hydrolases (autolysins), resulting in the weakening of the peptidoglycan meshwork. Here, we utilized E. coli strains NF314As19 (wild type relA), NF536As19 [relA valS(Ts)], and NF537As19 [relA valS(Ts)] as models to study the effects of the stringent response on ampicillin-induced lysis. This report confirmed that the strains behave appropriately by monitoring relative levels of RNA synthesis as an indicator of bacterial growth rate and the induction of the stringent response. This report also demonstrated a protective effect from ampicillin-induced lysis at 37°C produced from partially inactivating the ValS(Ts) protein. These results indicate that a partially activated stringent response in E. coli results in cellular processes that induce a degree of tolerance to ampicillin-induced lysis.

In their natural environment, bacteria are routinely challenged by stressful conditions such as nutrient limitation. Growth is closely linked with protein synthesis capability, and consequently the amount of cellular ribosomes. Upon amino acid deprivation, relA
Escherichia coli (E. coli) quickly accumulate intracellular guanosine 3',5'-bispyrophosphate (ppGpp), the nucleotide alarmone central to the adaptive phenomenon known as the stringent response (1, 3). Binding of ppGpp to RNA polymerase results in the coordinated inhibition of stable RNA transcription (e.g. rRNA and tRNA) and stimulation of amino acid biosynthesis gene transcription, together aimed at energy conservation (4).

Stringent response induction can initiate from starvation for an amino acid, which increases the cognate uncharged tRNA. When this uncharged tRNA binds to the ribosomal A site, ribosome-associated RelA catalyzes the production of (pp)pGpp and the stringent response is initiated (13). The resulting link between translation and transcription can be ‘relaxed’ (uncoupled) with a relA mutation; for example, RNA synthesis continues for more than 1 hr following translation inhibition (12). Peptidoglycan synthesis and β-lactam-induced lysis are also inhibited during the stringent response (6, 10). Inhibiting the action of autolysins (hydrolases) involved in construction of the peptidoglycan lattice may account for the penicillin tolerance phenomenon observed with amino acid-deprived relA (stringent) E. coli (2, 10). Relaxed (relA) E. coli do not accumulate ppGpp and consequently do not display features of stringent cells such as inhibited rRNA synthesis.

This experiment utilized three E. coli strains which allowed control of the stringent response through inactivation of a temperature-sensitive (Ts) amino acid biosynthetic gene: NF314As19 (wild type relA), NF536As19 [relA valS(Ts)], and NF537As19 [relA valS(Ts)]. Previously, it has been shown that similar RNA levels were achieved for strains NF536 and 537 at 42°C (5), a temperature previously thought to completely inactivate the ValS(Ts) protein, switching on the stringent response, and producing a decrease in RNA levels for stringent NF536 cells. Our report disproved these previous results, instead presenting a correct pattern of stringent and relaxed control for NF536 and NF537 respectively. We then tested whether the 37°C semi-permissive temperature (5), where NF536 should have a partial stringent response, would confer protection to ampicillin-induced lysis. It was hypothesized that at 37°C, elevated ppGpp levels produced from partial activation of the stringent response in NF536 would confer increased protection from ampicillin-induced lysis relative to NF537.

MATERIALS AND METHODS

Bacterial strains. E. coli NF314As19 (leu relA valS(Ts)), NF536As19 [leu relA valS(Ts)], and NF537As19 [leu relA valS(Ts)] strains were obtained from W. Ramey of the Department of Microbiology and Immunology, University of British Columbia. Complete inactivation of the temperature-sensitive valyl tRNA synthetase had been known to occur at 42°C, with 37°C being semi-restrictive, and 30°C being permissive (5).

Media and growth conditions. All strains were grown in M9 minimal media (Na2HPO4, 6 g/liter; KH2PO4, 3 g/liter; NaCl, 0.5 g/liter; NH4Cl, 1 g/liter; CaCl2, 3 mg/liter; MgSO4, 1 mM, pH 7.0), supplemented with 0.4% glucose, 1 ug/ml thiamine, and 50 ug/ml leucine to fulfill auxotrophic growth requirements. Cultures were grown in 30°C, 37°C, or 42°C shaking water baths with mild aeration.
FIG. 1 Analysis of growth characteristics for strains NF314, NF536 and NF537 grown at 30°C (A), 37°C (B), and 42°C (C).

at 250 rpm. Culture turbidity was measured with a Spectronic 20D spectrophotometer at 460 nm (OD460).

Strain preparation for uracil incorporation assays. Overnight cultures of the three NF strains were diluted 1-in-20 into 60 ml total using fresh media, then grown for 2 hours at 30°C, to obtain exponentially growing cells. The 60 ml culture was then split into 54 ml and 6 ml portions. Cold uracil (Sigma) was added to the 54 ml culture to give a final concentration of 54 ug/ml, then split into three 18 ml cultures, each being incubated at either 30°C, 37°C, or 42°C for growth curves. To the 6 ml culture, sufficient radioactive [14C]uracil was added to give 5 ug/ml final concentration, then split.
into three 2 ml cultures, and incubated at either 30°C, 37°C, or 42°C for scintillation counting.

Scintillation counting and turbidity readings. Before the 54 ml (cold) and 6 ml (radioactive) cultures were split, a turbidity reading was taken from the cold-uracil culture and 100 ul of radioactive uracil-treated culture was spotted onto a filter disc. After these two cultures were split, turbidity and filter-disc spotting were taken at time points 5, 10, 20, 30, 45, 60 minutes after incubations commenced at the three temperatures. The spotted filter discs were left to air dry, then immersed in 50 ml cold 5% trichloroacetic acid

FIG. 2 Radioactive [¹⁴C]uracil incorporation as an indicator of RNA synthesis. Strains NF314, NF536 and NF537 were grown, and sampled for incorporated [¹⁴C]uracil at different temperatures of 30°C (A), 37°C (B) and 42°C (C).
(TCA) solution. Ten minutes after adding the last sample, the used TCA was decanted, and 50 ml fresh cold 5% TCA was added. Ten minutes later, the TCA was decanted, followed by two 10 min 50 ml 95% ethanol washes. After the last ethanol wash, the filters were dried overnight at 95°C. Filters were then placed in vials with 3 ml scintillation fluid and read on a L5 6000 scintillation counter (Beckman).

**Results**

**Ampicillin-induced lysis.** Overnight cultures of NF356 and NF537 were diluted 1-in-20 with fresh media into 120 ml total volume, then incubated at 30°C with mild aeration until 0.4 OD_{600} was obtained. Each culture was then split, with one half going to 30°C, the other to 37°C. When 0.5 OD_{600} was reached, sufficient stock ampicillin (25 mg/ml, Sigma) was added to obtain a final concentration of 40 μg/ml. Cultures were monitored for β-lactam induced lysis and/or tolerance via OD_{600} readings.

**DISCUSSION**

In this experiment, we tested the effect of ampicillin on NF536 relA" cells under partial stringent control condition, in comparison to relaxed relA" mutants (NF537). Previous work with these strains had shown no decrease in RNA synthesis in NF536 cells under stringent control (5). This finding is contrary to previous work in regards to the effect of the stringent response on stable RNA synthesis (3): RNA synthesis decreases upon stringent response activation. Therefore, prior to performing ampicillin lysis assays, the temperature-sensitivity of ValS in NF356 and NF537 strains, as well as the relA" mutation in strain NF537, and NF536 RelA" activity had to be confirmed.

At 37°C and 42°C NF314 showed a considerably higher growth rate than the temperature sensitive strains, verifying the temperature sensitivity of NF536 and NF537 (Fig. 1B and Fig. 1C). The NF536 strain has drastically decreased growth at 42°C in comparison to the NF537 strain, implying stringent control activation in NF536 cells and RelA inactivity in NF537, under amino acid starvation conditions (Fig. 1C). The slightly lower growth rate of NF536 at 37°C, in comparison to NF537 cells grown at the same temperature (Fig. 1B), indicates the activation of a partial stringent response, as has previously been reported (5). At both temperatures the activity of stringent control in NF536 cells was confirmed with the uracil incorporation assay (Fig. 2).

It was expected that the uracil incorporation results should show a linear progression over time as radioactive uracil is incorporated into newly synthesized RNA. However, our results only show a linear progression for a 20-minute period after the addition of radioactive uracil after which point the curves begin to level (Fig. 2). This could be explained if the specific activity was too high so all the radioactive uracil was consumed within the first 20 minutes of the experiment and was unavailable to be incorporated into RNA synthesized after this point. The analysis of the data from the first 20 minutes is as expected and is consistent with the growth curve results. The decreased rate of RNA synthesis in the NF536 cultures at 37°C (Fig. 2B) indicates a higher level of cellular ppGpp expression (4) than the NF537 and NF314 strains, which can be attributed to the activation of a partial stringent response (5). There is a more drastic decrease in RNA synthesis at 42°C than 37°C (Fig. 2C), which would be consistent with even higher levels of cellular ppGpp and a stronger activation of a stringent control mechanism.

It has previously been established that amino acid deprived relA" cells exhibit tolerance to β-lactam
antibiotics such as penicillin, while relA mutants are susceptible to lysis upon treatment with these antibiotic (6). This tolerance is explained by the ability of the relA+ cells to activate the stringent response and, through the production of ppGpp, modulate peptidoglycan metabolism (9). We hypothesized that under conditions of partial stringent control, bacteria would show some tolerance to ampicillin since the effect of ppGpp on the process of peptidoglycan synthesis and β-lactam tolerance is concentration dependant (10). Rodionov and Ishiguro showed that the minimum concentration of ppGpp required to establish stringent control is about 70% of the maximum ppGpp level produced during the stringent response (10). Therefore, activation of a partial stringent response should result in production of enough cellular ppGpp to confer some tolerance to ampicillin. Therefore, upon treatment of NF536 and NF537 cell cultures growing at 37°C with ampicillin we would expect the NF536 relA+ stringent cells to show a delay (tolerance) in ampicillin-induced lysis while the NF537 relaxed strain should be affected more rapidly by ampicillin treatment.

We observed that for the NF536 strain grown at 30°C, ampicillin-induced lysis was characterized by a sharp drop in the optical density of the culture (Fig.
indicates a high level of sensitivity to antibiotic as cells lysed. This is expected since at this temperature stringent control is inactive and *E. coli* NF536 is susceptible to the effects of ampicillin. For NF536 cells growing at 37°C there was a considerably longer delay after the addition of ampicillin before a drop in optical density was observed (Fig. 3A and Table 1). Further, the drop in optical density and corresponding lysis rate was more gradual than seen with cells grown at 30°C (Fig. 3A and Table 1). This indicates that at 37°C the NF536 cells possessed some tolerance to the ampicillin treatment. This tolerance can be attributed to the partial activation of the RelA protein and production of ppGpp in cells. The ppGpp inhibits the activity of soluble lytic transglycosylase (*slt*), a key *E. coli* autolysin involved in the hydrolysis of peptidoglycan during the process of cell wall synthesis (2, 8). Both synthesis and hydrolysis of peptidoglycan are necessary for building the cell wall, and autolysins are therefore part of this normal activity (10). β-lactam antibiotics induce cell lysis by inhibiting the activity of polymerases [penicillin binding proteins (PBPs)] and thereby inhibiting peptidoglycan synthesis. Meanwhile, in continuing their regular function, the hydrolases/autolysins cause weakening of the peptidoglycan meshwork, resulting in lysis of the cell (11). Therefore, through inactivation of the autolysin activity, ppGpp appears to result in delayed lysis (tolerance) to β-lactams such as ampicillin.

For NF537, we would expect to see similar patterns of ampicillin induced lysis at 30°C and 37°C because at both temperatures the cells have low levels of ppGpp due to their permanently relaxed stringent control. At 37°C the ampicillin takes a longer time to induce lysis than at 30°C (Fig. 3B and Table 1). However, this observed delay is significantly shorter than was observed for the NF536 strain and is likely due to the fact that the antibiotic has a slower effect at lower (stringent) growth rates (Table 1). Therefore, tolerance that was exhibited by the NF536 was not observed for the NF537 strain. These results are in accordance with our expectation that the NF537 relaxed strain would display no tolerance to ampicillin-induced lysis because of the inability to activate the stringent response. The sharper drop in optical density observed in the case of the NF536 growing at 30°C was not observed for the NF537 growing at the same temperature (Fig. 3B and Table 1). This may be due to the slower action of ampicillin on the NF537 culture due to slower growth rates of this strain. Although we would expect the NF536 and NF537 strains to have similar growth rates, the NF537 strain showed a consistently higher doubling time at 30°C and 37°C than the NF536 strain at 30°C (Table 1). This implies that NF537 displays intrinsically slower growth than NF536 at non-stringent temperatures. This may be an indirect effect of the *relA* mutation in the NF537 strain.

The apparent leveling off of the lysis rate for NF537 at 37°C at time points at the end of sampled growth is puzzling (Fig. 3B). Our results imply that the NF537 37°C population is resistant relative to NF536, potentially implicating the loss of RelA in adaptation and survival to ampicillin. That is, after a large number of cells have been lysed, there is a subpopulation resistant to further lysis. This result is questionable. Further, this phenomenon has never before been reported in the literature. What is more likely is that our experiment at this point was limited due to insufficient culture, producing inaccurate turbidity readings. We expect that the curve would continue to decline to around the same turbidity reading as seen for NF536 at 37°C (Fig. 3A).

Overall, results obtained support our hypothesis that activation of a partial stringent response in *relA*+ cells confers some protection against ampicillin-induced

### TABLE 1 Characterization of the ampicillin-induced lysis stage in *E. coli*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Temperature (°C)</th>
<th>Doubling time (min)</th>
<th>Lag to lysis (min)a</th>
<th>Lysis rate (10^-2 -OD₆₀₀ per min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF536</td>
<td>30</td>
<td>80</td>
<td>40</td>
<td>2.4</td>
</tr>
<tr>
<td>(relA*)</td>
<td>37</td>
<td>160</td>
<td>100</td>
<td>0.3</td>
</tr>
<tr>
<td>NF537</td>
<td>30</td>
<td>120</td>
<td>50</td>
<td>1.1</td>
</tr>
<tr>
<td>(relA)</td>
<td>37</td>
<td>130</td>
<td>60</td>
<td>0.5</td>
</tr>
</tbody>
</table>

aTime from addition of ampicillin to decline in turbidity/lysis initiation.

3A), indicating a high level of sensitivity to antibiotic as cells lysed. This is expected since at this temperature stringent control is inactive and *E. coli* NF536 is susceptible to the effects of ampicillin.
lysis. Since the effect of ppGpp is concentration dependent, even through a partial stringent response relA+ cells can modulate the activity of the autolysins involved in peptidoglycan synthesis, thereby delaying autolysis. Delayed lysis was clearly seen in NF536 cultures displaying a partial stringent response when treated with ampicillin.

FUTURE EXPERIMENTS

Future experiments that require comparison of relA+ and relA mutant strains could include chloramphenicol treatment in a non-growing cell system. Chloramphenicol relaxes the stringent response (3), making a relA+ strain relaxed as though it had a relA mutation. Therefore, relA+ cells treated with chloramphenicol should behave similarly to relA mutant cells in terms of activation of the stringent response at conditions approaching non-growth. Adding this treatment would confirm that the difference in response shown by the relA+ and relA strains is in fact due to the activity of RelA and would also eliminate the problem of variability of response due to differences in growth rate.

The speed of the effect of the added ampicillin on growing cells was not determined. The unusual results observed in Fig. 3B could be resolved by sampling further time points to see if the curves begin to decline as predicted by Fig. 3A. This would confirm as to whether or not these results were real or inaccurate due to experimental limitation. Addition of non-antibiotic treated control cultures (e.g. no ampicillin) in our experimental system would verify that our observations are due to the effect of the antibiotic and not some other environmental or growth parameter. One could test the MIC of the strains to see if there is an intrinsic variation in antibiotic sensitivity.

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REFERENCES