Identification of a CysB-regulated gene involved in glutathione transport in Escherichia coli

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Abstract

Growth of Escherichia coli using the tripeptide glutathione as a sulfur source is well documented, but transport of glutathione into E. coli is uncharacterized. We have found that the ybiK gene, at 18.7 min, appears to be involved in the transport of glutathione and have therefore renamed ybiK as spt for sulfur peptide transport. The ybiK-lacZ fusion was found to be regulated by cysB, the transcriptional activator for the cysteine regulon. Mutations in the ybiK or cysA genes led to severe growth inhibition when cells were given glutathione as the sole sulfur source. In particular, strains of E. coli containing mutations in both the ybiK and cysA genes were unable to grow when the sole sulfur source provided was glutathione whereas single cysA mutants grew well with glutathione. In contrast, no such defects were seen when l-djenkolic acid or cysteine were used as the sole sulfur source. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Cysteine; Transcriptional regulation; Peptide transport; ybiK gene; cysB regulon

1. Introduction

Escherichia coli strains deficient in the genes of cysteine biosynthesis generally require an exogenous source of organic sulfur, such as cysteine, cystine, djenkolic acid (Cys-S-CH2-S-Cys), or glutathione, for growth [1]. Such organic sulfur sources are also required by cysA mutants due to their inability to transport sulfate or thiosulfate into the cell [2]. The tripeptide glutathione (γ-Glu-Cys-Gly) is widely used to protect cells against oxidative damage [3] and in E. coli can also be used as an osmoprotectant [4,5]. Glutathione synthesis depends on the glutathione synthetase genes gshAB; however, transport of exogenous glutathione into E. coli has not been characterized. Cysteine biosynthesis is controlled by the cysB gene. In the presence of the inducer N-acetyl-l-serine, CysB activates transcription of genes involved in sulfate and thiosulfate transport [2], sulfate reduction [1], and both inducer and cysteine synthesis [1,6]. In the presence of excess cysteine, inducer synthesis is inhibited, thereby decreasing both the activity of CysB and the transcription of the cysB gene itself [6–9].

The region of the E. coli chromosome from 18 to 19 min contains 41 genes, 31 of which are presently uncharacterized. While studying some of these genes, which we suspected of being induced under anaerobic conditions, we examined the regulation of the ybiK gene. We constructed an operon fusion of the ybiK gene to the reporter gene lacZ, encoding β-galactosidase, and found that expression of ybiK responded to the presence of cysteine and to defects in the ybiK gene, suggesting its involvement in sulfur metabolism. We also found that ybiK knockout mutations impaired the use of glutathione as sulfur source. The annotations of the ybiK sequence (accession number AE000185) in GenBank suggest that it is a putative amino acid utilization gene. Furthermore, GenBank suggests that the four genes downstream of ybiK may constitute a peptide transport operon and may be cotranscribed with ybiK. As a result of our findings we have renamed ybiK as spt for ‘sulfur peptide transport’ and suggest that the spt gene may encode a protein involved in glutathione transport or metabolism.
2. Materials and methods

2.1. Bacterial strains and culture media

All bacterial strains used were derived from *E. coli* K-12 and are described in Table 1. The *ybiK*spt::Kan strain AH69 was constructed in the lab of K.T. Shanmugam, during work on the characterization of the nearby molybdopterin synthesis genes *moeAB* [10]. AH69 contains partial deletions of *moeA* and its downstream neighbor, *ybiK*spt, with a kanamycin cassette placed in the remaining segment of *ybiKspt*. The minimal medium used was M9 [11] supplemented with 0.4% (w/v) carbon sources and 10 mg l⁻¹ vitamin B₁ when appropriate. Casamino acids were added to 0.1% (w/v). Difco Proteose Peptone #3 was added at 0.2%. Amino acids were added to 0.01% (w/v) unless otherwise indicated. Glutathione and 1-djenkolic acid were added to 1 mM. PPPS medium contained (per liter) 17 g of Bacto-peptone (Difco Laboratories, Detroit, MI, USA), 3 g of Difco Proteose Peptone #3, and 5 g NaCl. Solid media contained 1.5% (w/v) Difco Bacto-Agar. Anaerobic minimal media were supplemented with trace metals (50 μM Fe; 5 μM Mo; 5 μM Mn; 5 μM Zn; 5 μM Se). Aerobic cultures (20 ml) were grown in 125-ml side-arm conical flasks in a New Brunswick gyratory shaking water bath. Growth was followed by measuring the turbidity using a Klett-Summerson colorimeter equipped with a green (540-nm) filter. Anaerobic growth was carried out in anaerobic jars (Oxoid Ltd., London, UK) under an atmosphere of H₂/CO₂, which was generated by Oxoid Gas Generating kits. Resazurin indicators (Oxoid Ltd., London, UK) were used to ensure anaerobic conditions.

2.2. Enzyme assays

β-Galactosidase assays were performed as previously described [13]. The units of β-galactosidase activity are micromoles of o-nitrophenylgalactoside (ONPG) hydrolyzed per 10⁹ cells per hour, at 37°C. The results shown are averages of three independent assays, each performed in duplicate.

3. Results and discussion

3.1. Construction of *spt-lacZ* operon fusions

Mapping of some novel anaerobically induced genes to approximately 18 min [14] led us to further investigate several genes in the 18–19-min region of the *E. coli* chromosome. One of these genes, *ybiK*, which is located at 18.7 min, was originally suspected of being involved in anaerobic metabolism. We therefore constructed an operon fusion of the promoter of *ybiK*, now referred to as *spt*, to the *lacZ* structural gene using the method of Simons et al. [15]. The promoter of *spt* was inserted in front of the *lacZYA* genes carried on the vector pRS45 [15], giving *λJES2* (*sptl ybiK-lacZ*). Phage *λJES2* was then integrated into the λ-attachment site of *E. coli* strain MC4100, which contains a functional *spt* gene. β-Galactosidase assays performed...
on the spt-lacZ fusion strain, JJP17, revealed that spt (ybiK) was not induced under anaerobic conditions but was in fact expressed at slightly lower levels anaerobically (Table 2).

### 3.2. Transcriptional regulation of spt

Strains containing the spt-lacZ operon fusion were grown in both rich medium (PPPS) and minimal medium (M9). β-Galactosidase levels were lower when the cells were grown in PPPS medium than when grown in M9 minimal medium (Table 2) indicating that transcription of spt is lower when the medium is rich. In contrast, use of different carbon sources with M9 minimal medium (Table 2) showed negligible effect on spt-lacZ expression (Table 2). The effect of medium composition was further tested by growing the fusion strains in M9 minimal medium plus glucose with the addition of either Proteose Peptone #3 or casamino acids. Again, β-galactosidase expression was lower in the richer media (Table 2). As before, expression decreased approximately two- to three-fold in most cases when the strains were grown anaerobically, irrespective of the medium.

Since PPPS medium is composed mostly of amino acids and short peptides, we then tested the effects of the 20 common amino acids on the expression of spt-lacZ (Table 2). The presence of all 20 amino acids had a significant repressive effect on spt expression. Next, the amino acids were divided into two groups based upon their side chains, referred to in Table 2 as set 1 and set 2. Set 2 caused a two- to three-fold decrease in expression of spt and was further subdivided into basic, aromatic, and sulfur-containing (Table 2). Only the sulfur-containing amino acids cysteine and methionine affected expression significantly. Cysteine was found to have the biggest effect on the expression of spt, whereas methionine had a smaller effect, perhaps due to recycling of sulfur from methionine via homocysteine. In GenBank, spt (ybiK) (accession number AE000185) is annotated as a putative asparaginase. Therefore, the strains containing the fusion were grown in M9 glucose medium plus asparagine and aspartate to determine if these amino acids had any effect. However, the combination of asparagine and aspartate had no significant effect on spt-lacZ expression (Table 2).

Since spt transcription was repressed by cysteine, it seemed possible that spt would be regulated by cysB, the transcriptional regulatory gene for the cysteine regulon. If so, a defect in cysB should result in decreased expression of spt. To test this, a cysB mutation was moved into the spt-lacZ fusion strain, JJP17, to give JJP48. E. coli strains defective in cysB require an exogenous organic sulfur source for growth. Because cysteine down-regulates spt, l-djenkolic acid and glutathione were used as alternative sulfur sources when testing cysB mutants (Table 3). Neither l-djenkolic acid nor glutathione had any significant effect on expression of spt. However, in the presence of the cysB mutation, expression of spt was lower regardless of sulfur source, implying that cysB is indeed a transcriptional activator of spt. Insertional inactivation of spt itself was also tested on expression of the spt-lacZ fusion (Table 3). The spt-deficient strain had unaltered levels of spt-lacZ expression indicating that spt is not autoregulated. In addition, a number of other gene defects were tested for possible effects on spt expression. Insertional inactivation of moe, glnG, glnA, and lrp had no effect on spt expression (data not shown).

### Table 3

Expression of spt-lacZ fusion in strains containing either an spt or a cysB mutation

<table>
<thead>
<tr>
<th>Strain</th>
<th>β-Galactosidase activity</th>
<th>cysteine</th>
<th>l-djenkolic acid</th>
<th>glutathione</th>
</tr>
</thead>
<tbody>
<tr>
<td>JJP17</td>
<td>6198</td>
<td>1574</td>
<td>3727</td>
<td>7850</td>
</tr>
<tr>
<td>JJP48 cysB</td>
<td>–</td>
<td>453</td>
<td>491</td>
<td>2297</td>
</tr>
<tr>
<td>JJP19 spt</td>
<td>6231</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

All strains contain the spt-lacZ fusion. Strains were grown in M9 minimal medium plus 0.4% glucose with the addition of a final concentration of 1 mM MgSO₄, cysteine, l-djenkolic acid, or glutathione. Units of β-galactosidase activity are micromoles of ONPG hydrolyzed per 10⁶ cells per hour at 37°C. JJP48 cannot grow with sulfate as sole sulfur source, so no data were obtained.
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Table 4
Growth properties of mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Sulfur source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>None added</td>
</tr>
<tr>
<td>MC4100</td>
<td>parent</td>
<td>++++</td>
</tr>
<tr>
<td>JJP155 nupC::Tn10</td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>HS240 cysA nupC::Tn10</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>JJP154 cysA nupC::Tn10</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>JJP19 Δspt::Kan</td>
<td></td>
<td>++++</td>
</tr>
<tr>
<td>JJP151 Δspt::Kan nupC::Tn10</td>
<td></td>
<td>++++</td>
</tr>
<tr>
<td>JJP150 Δspt::Kan cysA nupC::Tn10</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>JJP48 cysB</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>JJP148 cysB Δspt::Kan</td>
<td></td>
<td>–</td>
</tr>
</tbody>
</table>

All strains were grown on M9 minimal medium agar containing 0.4% glucose and 1 mM MgSO₄, L-djenkolic acid, or glutathione. All strains were incubated at 37°C for 1 day. Growth was scored visually, with ++++ being heavy growth to ^ being no growth.

3.3. Effects upon growth of mutations in cysB and spt

In GenBank, spt/lybK is listed as the first gene of a putative five-gene operon possibly involved in peptide transport. We therefore tested the growth properties of strains deficient in spt (Table 4) versus a variety of sulfur sources, including the sulfur-containing tripeptide, glutathione. The Δspt::Kan insertion alone, as in strain JJP19, showed little effect on growth. However, wild-type E. coli can grow on the traces of sulfate in agar, even in the absence of an added sulfur source. This was confirmed by inoculating the parental strain, MC4100, and JJP19 onto M9 minimal agar lacking added sulfate. Both strains grew the same without sulfate as with 1 mM MgSO₄ (Table 4). Mutations in cysA abolish transport of sulfate or thiosulfate into the cell and thus prevent growth on agar containing MgSO₄ or traces of sulfate. Therefore, a mutation in the cysA gene was introduced to eliminate growth due to sulfate contamination. The presence of a cysA mutation alone (as in HS240 or JJP154) prevented growth with sulfate but had no effect on the use of glutathione as sulfur source. The presence of nupC::Tn10, used for cotransduction of cysA, had no effect on growth with any sulfur source (Table 4).

The cysA mutation was then transduced into the spt::Kan knockout strain JJP19. This almost completely abolished growth on glutathione as seen for the double mutant, JJP150, suggesting that the spt operon is involved in glutathione transport. This effect is due to spt, since single cysA mutants grew well on glutathione. The apparent growth of the spt::Kan strain JJP19 on glutathione was thus presumably due to the presence of traces of sulfate in the agar. In addition, the cysB mutant strain JJP48 showed greatly decreased growth on glutathione (Table 4). This agrees with the finding that spt is under the positive control of CysB and would be non-functional in cysB strains. All strains were able to grow with cysteine as sulfur source (data not shown) and growth on L-djenkolic acid did not appear to be significantly affected by spt and/or cysA.

4. Conclusion

Transcriptional lacZ gene fusions were constructed to the promoter of spt (formerly known as ybiK) and were found to be down-regulated in the presence of excess amino acids. Further examination identified cysteine as the effective component. This effect was attributed to CysB, the transcriptional activator of the cysteine regulon. High levels of intracellular reduced sulfur, such as cysteine, cause a decrease in the transcription of cysB, which in turn decreases the transcription of genes in the cys regulon [1]. This was shown for spt by introducing a mutation in cysB, which caused a decrease in the expression of spt–lacZ. Comparison of the cysA mutant strain JJP154, which grew well on glutathione as sole sulfur source, and the cysA spt double mutant JJP150, which grew very poorly on glutathione, suggested that the spt gene was involved in glutathione transport.

GenBank annotations suggest that splybK (accession number AE000185) is a putative amino acid utilization gene and that the gene immediately downstream of spt is a hypothetical transport protein. Furthermore, the next three downstream genes are similar to the dipeptide permease (dppABC) genes [16] of E. coli. GenBank sequence data indicates that these five genes may be cotranscribed. Taken together with our data, this suggests that spt is involved in transport of peptides, including glutathione. Thus, we renamed ybiK as spt for sulfur peptide transport. Whether or not this system can transport other sulfur-containing peptides, such as glutathione conjugates, is unknown.

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References


