Temperature-sensitive mutation in \textit{ltyF}, a new gene involved in autolysis of \textit{Escherichia coli}

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A temperature-sensitive mutation in a new \textit{Escherichia coli} gene, located at 62.5 min on the linkage map and designated \textit{ltyF}, resulted in bacteriolysis at the restrictive temperature. Temperature sensitivity and \textit{ltyF}-mediated lysis were simultaneously suppressed by either of two previously described unlinked mutations designated \textit{smhA1} and \textit{smhB1}. The \textit{smhA1} and \textit{smhB1} alleles were originally isolated as specific extragenic suppressors of temperature-sensitive mutations in three other genes known as \textit{murH} (99 min), \textit{ltyD} (13 min) and \textit{ltyE} (25 min) which conferred lysis phenotypes indistinguishable from that of the \textit{ltyF} mutation. The \textit{murH}, \textit{ltyD} and \textit{ltyE} genes have been proposed to be related on the bases of phenotypic similarities and the specificities of their extragenic suppressors. It is now further proposed that \textit{ltyF} belongs to this group. The isolation of new alleles of \textit{smhA} and \textit{smhB} as extragenic suppressors of \textit{ltyF} further supports this proposal.

Introduction

The bacterial peptidoglycan sacculus is a closed structure. Therefore, the cleavage of covalent bonds within this structure by peptidoglycan hydrolases is thought to be necessary for the expansion of the sacculus and for septation in growing bacteria. At least nine peptidoglycan hydrolases have been demonstrated in \textit{Escherichia coli} (reviewed by Hölétje & Tuomanen, 1991, and by Hölétje & Schwarz, 1985). The exact functions of these enzymes and the modes by which their activities are regulated are poorly, if at all, understood.

We have previously described temperature-sensitive mutations in three unlinked genes designated \textit{murH} (99 min on the \textit{E. coli} chromosomal linkage map), \textit{ltyD} (13 min) and \textit{ltyE} (25 min) which confer bacteriolytic phenotypes at the restrictive temperature (Dai & Ishiguro, 1988, 1990, 1991a). The lysis associated with each of these mutations is mediated by a peptidoglycan hydrolase as evidenced by the solubilization of radiolabelled peptidoglycan. The mutations are suppressed by secondary mutations in either of two genes called \textit{smhA} and \textit{smhB}. The suppressor activities of the \textit{smhA1} and \textit{smhB1} alleles are evidently specifically directed toward mutations in \textit{murH}, \textit{ltyD} and \textit{ltyE}, e.g. they do not suppress the lysis resulting from the blockage of peptidoglycan synthesis through either antibiotic treatment or mutation in a peptidoglycan biosynthetic enzyme. We have therefore proposed that these genes are related because of the apparent specificity of their extragenic suppressors, and they are referred to hereafter as the \textit{murH} group.

In view of recent results discussed below, it is relevant to note that the \textit{ltyD1} mutation is suppressed by cloned multicopies of either the \textit{ci} or the \textit{cro} genes of bacteriophage \textit{\lambda} (Dai & Ishiguro, 1991b). \textit{Ci} and \textit{Cro} are \textit{\lambda} DNA-binding proteins, both of which compete for the same right operator sequences of the \textit{\lambda} genome. We have therefore proposed that \textit{LytD} may be a DNA-binding protein with a specificity similar to that of \textit{Ci} and \textit{Cro}. \textit{LytD} may be a transcriptional repressor of a lysis gene.

In this paper, we describe still another unlinked gene, designated \textit{ltyF} (located at 62.5 min), belonging to the \textit{murH} group. A mutation in \textit{ltyF}, like mutations in other members of the \textit{murH} group, conferred temperature sensitivity and the rapid onset of lysis at the restrictive temperature.

Methods

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Table 1. Escherichia coli K-12 strains

<table>
<thead>
<tr>
<th>Strain</th>
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<tr>
<td>JC158</td>
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<td>VC7</td>
<td>thi−1 lysA23 rpsL109</td>
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<td>VC7 except zaa−1::Tn5 murH1</td>
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<td>VC1110</td>
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<td>This study</td>
</tr>
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<td>VC4120</td>
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<td>Dai &amp; Ishiguro (1991a)</td>
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<td>W3110</td>
<td>Prototroph</td>
<td>CGSC</td>
</tr>
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</table>

*CGSC: Dr Barbara Bachmann, E. coli Genetic Stock Center, Yale University, New Haven, CT, USA.

Minimal Agar (Difco) with appropriate supplements was used for selection of auxotrophic markers.

**Bacterial strains and isolation of the lytFl mutant.** The E. coli K-12 strains used in this study are described in Table 1. The transposon insertions, zee−102::Tn10, zee−1::Tn10kan, and zbc−103::Tn10 were used to facilitate the construction of strains carrying alleles of smhA and smhb as described previously (Dai & Ishiguro, 1990, 1991a). The lysA30::Tn10 allele used for mapping of lytFl was from our laboratory collection.

The lytFl mutant was isolated during an intensive and systematic search in our laboratory for new mutations affecting peptidoglycan metabolism. We employed a modified version of localized mutagenesis (Hong & Ames, 1971) for this purpose. Strain W3110 was mutagenized with ethylmethane sulphonate (EMS) as described by Miller (1972). The EMS-mutagenized culture was screened for temperature-sensitive mutations in specific chromosomal locations by P1vir-mediated transduction. For example, the lytFl mutation was discovered in a screen for mutations in the 63 min region of the E. coli linkage map. This was accomplished by transducing strain JC158 (serA) to serA* at 30 °C with a bacteriophage P1vir lysate prepared on the EMS-mutagenized W3110 culture. The serA* transductants were then screened for temperature sensitivity, i.e. inability to grow at 42 °C. The physiological and morphological properties of the temperature-sensitive derivatives were determined, and those with apparent defects in cell envelope function were retained. The lytFl mutant, strain VC1100, was chosen for detailed study because it exhibited lysis during incubation at the restrictive temperature.

**Culture conditions.** Bacteria were grown in Tryptic Soy Broth (TSB, Difco) or Nutrient Broth (NB, Difco). Minimal Agar (Difco) with appropriate supplements was used for selection of auxotrophic markers. TSB was added to NA and NB at 1% (w/v) where indicated. Where required, tetracycline and kanamycin were used at 20 and 50 µg ml−1, respectively. Cultures in liquid media were incubated in waterbath shakers at the indicated temperatures, and growth was monitored in a Klett-Summerson colorimeter with a green filter.

**Table 1. Escherichia coli K-12 strains**

- **Strain**: JC158, VC7, VC460, VC1110, VC1137, VC1138, VC1140, VC1142, VC1144, VC1146, VC1148, VC1149, VC1188, VC4077, VC4120, VC4229, W3110
- **Genotype**: Various, including mutations
- **Source**: CGSC, Laboratory collection, Dai & Ishiguro (1988), Dai & Ishiguro (1990), Dai & Ishiguro (1991a)

Fig. 1. Temperature-dependent lysis of strain VC1110 as determined turbidimetrically (a) and by solubilization of radiolabelled peptidoglycan (b). VC1110 was grown at 30 °C for two doublings before being divided into two portions at 0 min. One portion was incubated at 30 °C (○) and the other at 42 °C (●).

NaCl. One set of plates was incubated at 30 °C and the other at 42 °C. Plate counts were determined after 48 h of incubation. Incubation for longer periods did not change the plate counts. The effect of temperature on colony formation, or plating efficiency, was expressed as the ratio of the plate count (in terms of c.f.u. ml−1) obtained at 42 °C to the plate count obtained at 30 °C.

**Solubilization of radiolabelled peptidoglycan.** Peptidoglycan was labelled with [G-3H]diaminopimelic acid ([3H]DAP, Amersham) as described previously (Dai & Ishiguro, 1988). Strain VC1110 was grown in TSB to a density of 2 × 10^8 cells ml−1. [3H]DAP was then added to a final concentration of 0.6 µg ml−1 (22 µCi µl−1). After one doubling, unlabelled DAP was added at 25 µg ml−1 to terminate the labelling. The labelled culture was divided into two portions (see Fig. 1). One portion was incubated at 30 °C and the other portion was shifted to 42 °C. At the indicated times, 0.5 ml samples were added to an equal volume of 4% (w/v) SDS and boiled for 30 min. The SDS-insoluble fractions were collected on Millipore filters (pore size 0.45 µm). The
filters were rinsed with distilled water, dried and counted in a Beckman LS3145T liquid scintillation counter in a toluene-based scintillation cocktail (Dai & Ishiguro, 1988).

Isolation of extragenic suppressor mutants. Extragenic mutations which suppressed lytF1 were selected by two procedures originally designed for the isolation of murH1 suppressors. In the first method (Dai & Ishiguro, 1991a), suppressors which restored colony formation at 42 °C were selected. Strain VC1137 was plated on TSA, and colonies appearing after overnight incubation at 42 °C were picked for further characterization. As noted below, this method resulted in the isolation of new smhA alleles. In the second method (Dai & Ishiguro, 1990), we selected mutations which apparently permitted survival of the lytF1 mutant, strain VC1142, during overnight incubation on TSA at 42 °C but did not support colony formation under these conditions. Colonies of such suppressor mutants became apparent only after the plates were downshifted to 30 °C for an additional period of about 24 h. As noted below, the suppressor mutations obtained by this second method represented new alleles of smhB.

Reproducibility of results. Every experiment was performed twice. All experiments were found to be completely reproducible, and representative results are presented.

Results and Discussion

Phenotypic and genetic characterization of lytF1

Strain VC1110 exhibited temperature-sensitive growth and failed to form colonies at 42 °C on a variety of media. This temperature sensitivity was correlated with a bacteriolytic phenotype at the restrictive temperature. When cultures growing in either complex (Fig. 1a) or minimal (data not shown) media were subjected to 30–42 °C temperature upshifts, lysis occurred within one doubling time. Lysis coincided with the solubilization of cell wall peptidoglycan which had been prelabelled with [3H]DAP (Fig. 1b) and therefore indicated the involvement of a peptidoglycan hydrolase. About 90% of the radiolabelled peptidoglycan was solubilized within 2 h.

In linkage mapping experiments with bacteriophage P1, the mutation in strain VC1110 was cotransducible with the serA and lysA loci at frequencies of 48% and 11%, respectively. As shown in Fig. 2, it was assigned to 62-5 min on the E. coli linkage map (Bachmann, 1990). There are no known genes in this area which could obviously account for the phenotype of VC1110. Therefore, the mutation apparently represented a new locus and was designated lytF.

The lytF1 phenotype of VC1110 resembled the phenotypes of three previously described related mutant alleles designated murH1 (Dai & Ishiguro, 1988), lytD1 (Dai & Ishiguro, 1990) and lytE1 (Dai & Ishiguro, 1991a). The mutations in this group are specifically suppressed by mutations in either the smhA or the smhB genes (Dai & Ishiguro, 1990, 1991a). To test the possible relationship of lytF1 with the murH group further, we constructed lytF1 derivatives carrying either the smhA1 or the smhB1 mutations.

Suppression of lytF1 by smhA

The smhA1 mutation suppressed the lytic phenotype associated with the lytF1 mutation in both NB+ 1% NaCl (Fig. 3a) and NB (Fig. 3b) in comparative studies with the isogenic strains, VC1137 (smhA+EytF1) and VC1138 (smhA1 lytF1) and was particularly effective in high osmolarity media such as NB+1% NaCl. Although smhA1 suppressed lytF1-mediated lysis, it clearly had a detrimental effect on growth at the permissive temperature (30 °C) when combined with lytF1, especially in low osmolarity media. This is evident in Fig. 3(b) where strain VC1137 is shown to exhibit a doubling time of about 50 min in NB at 30 °C; in contrast VC1138 had a doubling time of over 100 min with a significantly lower final cell yield. In this regard, it is notable that smhA1 by itself did not confer an obvious phenotype and did not noticeably affect cell growth. However, in previous studies (Dai & Ishiguro, 1991a), we suspected that smhA1, in some subtle way represented a handicap. We noted that spontaneous mutations in lytE arose at high frequency during the routine laboratory maintenance of the smhA1 mutant. It seemed unlikely that this was due to coincidence, and we consequently suggested that the spontaneous lytE mutations may serve to suppress a detrimental effect of smhA1 that was not obvious in strains carrying this mutation alone (Dai & Ishiguro, 1991a). Thus, the suspected detrimental effect of smhA1 was demonstrated here for the first time in a lytF1 background.

The temperature sensitivity of strain VC1137 (smhA+EytF1) on both high and low osmolarity media is documented in Table 2. In contrast, temperature sensitivity on NA+1% NaCl was completely abolished by smhA1 in strain VC1138. Furthermore, smhA1 also partially restored colony formation on NA as evidenced by the 1000-fold difference in the plating efficiencies of VC1137 and VC1138. This partial suppressor activity of smhA1 was probably a reflection of the effects of smhA1 on growth in NB (Fig. 3b).

![Fig. 2. Linkage mapping of lytF by phage P1vir-mediated transduction with VC1110 and JC158 as the donor and recipient strains, respectively. The arrowheads indicate the positions of the unselected markers, and linkages are expressed as percentage cotransduction. Over 200 transductants were analysed in each determination.](image-url)
Temperature-resistant derivatives of strain VC1110 arose at a frequency of about $3 \times 10^{-6}$. Five independent temperature-resistant isolates were chosen for genetic characterization. All five were shown to carry extragenic suppressors of lytF which were cotransducible with zce-102::Tn10 and zce-1::Tn10kan at frequencies of 56% and 60%, respectively. Therefore, their genetic map positions coincided with that of smhAI. We have so far been unable to develop a system for complementation analysis of this region. Nevertheless, we have tentatively assigned these new alleles to the smhA locus because the new alleles conferred the same phenotypic characteristics as smhAI. We have so far been unable to develop a system for complementation analysis of this region. Nevertheless, we have tentatively assigned these new alleles to the smhA locus because the new alleles conferred the same phenotypic characteristics as smhAI. Thus, smhA8 restored temperature-resistant colony formation in a lytFI background on NA+1% NaCl but not on NA (strain VC1149, Table 2). Furthermore, smhA8, like smhAI (Dai & Ishiguro, 1991a), suppressed the temperature sensitivities of a murH1 mutant (strain VC1188, Table 2) and a lytE1 mutant (strain VCVC4229, Table 2) on both high and low osmolarity media. It is also significant that extragenic suppression of lytFI seemed to be restricted to a single locus, smhA. The same observation has been made with other members of the murH group (Dai & Ishiguro, 1991a).

In summary, two findings suggest that lytF is a member of the murH group: (i) the lytFI mutant could be used to isolate new smhA alleles; and (ii) the smhA alleles suppressed lytE1, lytFI and murH1.

**Suppression of lytFI by smhB**

Table 3 confirms our earlier observation (Dai & Ishiguro, 1990), indicating that the smhBI mutation, by itself, exhibits what has been termed by Csonka (1989) an osmoremedial temperature-sensitive phenotype. Thus, strain VC1144 (smhBI lytFI) formed colonies at 42°C on a high osmolarity medium such as NA+1% NaCl but not on a low osmolarity medium such as NA (Table 3). We have further shown that the suppression of murH1-mediated lysis by smhBI is independent of medium osmolarity (Dai & Ishiguro, 1990). Fig. 4(a) shows that smhBI suppressed lytFI-mediated lysis in the
same way. A comparison of the isogenic strains, VC1140 (smhB1 lytF1) and VC1142 (smhB+ lytF1), grown in NB and NB + 1% NaCl at 42 °C, clearly demonstrates that lysis suppression by smhB1 was independent of osmolarity. Although lysis was suppressed in low osmolarity media, VC1140 still failed to form colonies at 42 °C on NA (Table 3), and this undoubtedly reflected the growth restriction imposed under these conditions by the smhB1 mutation (i.e. see VC1144 in Table 3) in this strain. This restricted growth, expected in NB, was not obvious in the experiment described in Fig. 4(b) probably because of its relatively short duration. Exactly the same results were obtained with a smhB1 murH1 mutant strain (Dai & Ishiguro, 1990).

We subjected the lytF1 mutant strain, VC1142, to the procedure used previously (Dai & Ishiguro, 1990) to isolate the smhB1 derivative. By this method, we were readily able to isolate additional suppressor mutants, all of which carried alleles that were assigned to the smhB locus on the bases of genetic linkage mapping and phenotypic characterization. Table 3 shows that one such example, smhB3, exhibited the characteristic osmoresistant temperature sensitivity (strain VC1148) and restored temperature-resistant colony formation to a lytF1 derivative (strain VC1146). Furthermore, the smhB3 allele suppressed lytF1-mediated lysis in both high and low osmolarity media (Fig. 4b).

Together, the ability of smhB1 to suppress lytF1 and the use of the lytF1 mutant as a means of isolation of additional smhB alleles represent still further evidence for the relationship between lytF and the murH group.

Recent observations on the basis for murH-mediated lysis and relationship to lytF

The lytic phenotypes associated with the murH group of mutations resembled those of previously described mutants blocked in various steps of peptidoglycan synthesis (e.g. Lugtenberg & van Schijndel-Van Dam, 1972a, b, 1973; Matsuzawa et al., 1969; Salmond et al., 1980). Our attempts to associate these mutations with defects in specific steps of peptidoglycan biosynthesis have so far been unsuccessful. However, we have recently demonstrated that the peptidoglycan hydrolase activity associated with murH1-mediated lysis is encoded by an unidentified cryptic prophage which is unrelated to phage λ as determined by DNA hybridization; we have also shown that the activation of this hydrolase activity at the restrictive temperature is a specific consequence of the murH1 mutation (E. E. Ishiguro, unpublished data). With the discovery of lytF, the murH group now comprises four lysis-inducing mutations. Because they are unlinked, we are currently attempting to determine whether the various mutations activate the same prophage-encoded peptidoglycan hydrolase. How the smhA and smhB mutations suppress peptidoglycan hydrolase activation is also of interest.

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References