Temperature-sensitive mutation in *lytF*, a new gene involved in autolysis of *Escherichia coli*

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A temperature-sensitive mutation in a new *Escherichia coli* gene, located at 62.5 min on the linkage map and designated *lytF*, resulted in bacteriolysis at the restrictive temperature. Temperature sensitivity and *lytF*-mediated lysis were simultaneously suppressed by either of two previously described unlinked mutations designated *smhA1* and *smhB1*. The *smhA1* and *smhB1* alleles were originally isolated as specific extragenic suppressors of temperature-sensitive mutations in three other genes known as *murH* (99 min), *lytD* (13 min) and *lytE* (25 min) which conferred lysis phenotypes indistinguishable from that of the *lytF* mutation. The *murH*, *lytD* and *lytE* 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 *lytF* belongs to this group. The isolation of new alleles of *smhA* and *smhB* as extragenic suppressors of *lytF* 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 *Escherichia coli* (reviewed by Høltje & Tuomanen, 1991, and by Høltje & 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 *murH* (99 min on the *E. coli* chromosomal linkage map), *lytD* (13 min) and *lytE* (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 radio-labelled peptidoglycan. The mutations are suppressed by secondary mutations in either of two genes called *smhA* and *smhB*. The suppressor activities of the *smhA1* and *smhB1* alleles are evidently specifically directed toward mutations in *murH*, *lytD* and *lytE*, 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 *murH* group.

In view of recent results discussed below, it is relevant to note that the *lytD1* mutation is suppressed by cloned multicopies of either the *cI* or the *cro* genes of bacteriophage λ (Dai & Ishiguro, 1991b). *CI* and *Cro* are λ DNA-binding proteins, both of which compete for the same right operator sequences of the λ genome. We have therefore proposed that *LytD* may be a DNA-binding protein with a specificity similar to that of *CI* and *Cro*. *LytD* may be a transcriptional repressor of a lysis gene.

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

Methods

Genetic techniques. Bacteriophage P1vir-mediated generalized transduction was performed according to the methods of Miller (1972). Genetic linkages were calculated as described by Wu (1966). Davis

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Table 1. *Escherichia coli* K-12 strains

Strain	Genotype	Source*
JC158	<i>lacI22</i> λ^- <i>relA1 serA6 spoT1 thi-1</i>	CGSC
VC7	<i>thi-1 lysA23 rpsL109</i>	Laboratory collection
VC460	VC7 except <i>zsa-1::Tn5 murH1</i>	Dai & Ishiguro (1988)
VC1100	JC158 except <i>serA⁺ lytF1</i>	This study
VC1110	VC1100 except <i>lysA 30::Tn10</i>	This study
VC1137	VC1100 except <i>zce-102::Tn10</i>	This study
VC1138	VC1100 except <i>zce-102::Tn10 smhA1</i>	This study
VC1140	VC1100 except <i>zbc-103::Tn10 smhB1</i>	This study
VC1142	VC1100 except <i>zbc-103::Tn10</i>	This study
VC1144	VC7 except <i>zbc-103::Tn10 smhB1</i>	This study
VC1146	VC1142 except <i>smhB3</i>	This study
VC1148	VC7 except <i>zbc-103::Tn10 smhB3</i>	This study
VC1149	VC1137 except <i>smhA8</i>	This study
VC1188	VC460 except <i>zce-102::Tn10 smhA8</i>	This study
VC4077	VC7 except <i>zbc-103::Tn10 smhB1</i>	Dai & Ishiguro (1990)
VC4120	VC7 except <i>zce-102::Tn10 smhA1</i>	Dai & Ishiguro (1991a)
VC4229	VC7 except <i>zce-102::Tn10 lytE1 zce-1::Tn10kan smhA 8</i>	This study
W3110	Prototroph	CGSC

* 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 *lytF1* mutant. The *E. coli* K-12 strains used in this study are described in Table 1. The transposon insertions, *zce-102::Tn10*, *zce-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 *lytF1* was from our laboratory collection.

The *lytF1* 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 *lytF1* 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 *lytF1* 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). Solidified versions of these media, TSA and NA, respectively, contained 1.5% (w/v) agar. NaCl 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. To assess the temperature sensitivities of the strains listed in Tables 2 and 3, cultures were grown to stationary phase in TSB at 30 °C. Serial dilutions of the cultures were plated in duplicate on NA and NA containing 1% (w/v)

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 (2.2 µCi µg⁻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 boiling 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*⁺ *lytF1*) 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*⁺ *lytF1*) 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.

Fig. 3. Growth of strains VC1137 (*smhA*⁺ *lytF*1; ○, ●) and VC1138 (*smhA*1 *lytF*1; △, ▲) in NB+1% NaCl (a) and NB (b). In both experiments, the cultures of VC1137 and VC1138 were grown for two doublings at 30 °C. At 0 min, each culture was divided into two portions, and one of these portions was kept at 30 °C (open symbols) whilst the other was shifted to 42 °C (closed symbols).

Table 2. Colony formation by isogenic *smhA* *lytF* and *smhA* *murH* strains as a function of growth media and temperature

Strain	Plating efficiency	
	NA	NA + 1% NaCl
VC1137 (<i>smhA</i> ⁺ <i>lytF</i> 1)	2.5×10^{-8}	6.4×10^{-5}
VC1138 (<i>smhA</i> 1 <i>lytF</i> 1)	2.5×10^{-5}	0.9
VC1149 (<i>smhA</i> 8 <i>lytF</i> 1)	1.4×10^{-5}	0.9
VC1188 (<i>smhA</i> 8 <i>murH</i> 1)	1.0	1.0
VC4229 (<i>smhA</i> 8 <i>lytE</i> 1)	0.9	0.9

Temperature-resistant derivatives of strain VC1110 arose at a frequency of about 3×10^{-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 *smhA*1. 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 *smhA*1. Thus, *smhA*8 restored temperature-resistant colony formation in a *lytF*1 background on NA+1% NaCl but not on NA (strain VC1149, Table 2). Furthermore, *smhA*8, like *smhA*1 (Dai & Ishiguro, 1991a), suppressed the temperature sensitivities of a *murH*1 mutant (strain VC1188, Table 2) and a *lytE*1 mutant (strain VCVC4229, Table 2) on both high and low osmolarity media. It is also significant that extragenic

Table 3. Colony formation by *smhB* and *lytF* strains as a function of growth media and temperature

Strain	Plating efficiency	
	NA	NA + 1% NaCl
VC1140 (<i>smhB</i> 1 <i>lytF</i> 1)	5.8×10^{-7}	0.6
VC1142 (<i>smhB</i> ⁺ <i>lytF</i> 1)	1.0×10^{-5}	5.2×10^{-5}
VC1144 (<i>smhB</i> 1 <i>lytF</i> ⁺)	1.2×10^{-6}	1.1
VC1146 (<i>smhB</i> 3 <i>lytF</i> 1)	5.1×10^{-8}	1.0
VC1148 (<i>smhB</i> 3 <i>lytF</i> ⁺)	5.8×10^{-7}	0.9

Fig. 4. (a) Growth of strains VC1142 (*smhB*⁺ *lytF*1; ○, ●) and VC1140 (*smhB*1 *lytF*1; △, ▲) at 42 °C in NB (open symbols) and NB+1% NaCl (closed symbols). (b) Growth of strains VC1142 (*smhB*⁺ *lytF*1; ○, ●) and VC1146 (*smhB*3 *lytF*1; △, ▲) at 42 °C in NB (open symbols) and NB+1% NaCl (closed symbols). The cultures were grown at 30 °C for two doublings and then shifted to 42 °C at 0 min.

suppression of *lytF*1 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 *lytF*1 mutant could be used to isolate new *smhA* alleles; and (ii) the *smhA* alleles suppressed *lytE*1, *lytF*1 and *murH*1.

Suppression of *lytF*1 by *smhB*

Table 3 confirms our earlier observation (Dai & Ishiguro, 1990), indicating that the *smhB*1 mutation, by itself, exhibits what has been termed by Csonka (1989) an osmoremedial temperature-sensitive phenotype. Thus, strain VC1144 (*smhB*1 *lytF*⁺) 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 *murH*1-mediated lysis by *smhB*1 is independent of medium osmolarity (Dai & Ishiguro, 1990). Fig. 4(a) shows that *smhB*1 suppressed *lytF*1-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 osmo-remedial 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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