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Overexpression of a delayed early gene *hlg1* of temperate mycobacteriophage L1 is lethal to both *M. smegmatis* and *E. coli*

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Two genes of temperate mycobacteriophage L5, namely, *gp63* and *gp64*, were hypothesized to be toxic to *M. smegmatis*. An identical L5 *gp64* ortholog (designated *hlg1*) was cloned from homoimmune mycobacteriophage L1 and characterized at length here. As expected, *hlg1* affected the growth of *M. smegmatis* when overexpressed from a resident plasmid. HLG1 (the protein encoded by *hlg1*) in fact caused growth retardation of *M. smegmatis* and the region encompassing its 57-114 C-terminal amino acid residues was found indispensable for its growth-retardation activity. Both nucleic acid and protein biosynthesis were severely impaired in *M. smegmatis* expressing HLG1. Interestingly, HLG1 also affected *E. coli* almost similarly. This putative delayed early lipoprotein did not participate in the lytic growth of L1. [BMB reports 2008; 41(5): 363-368]

INTRODUCTION

Bacteriophages usually utilize bacterial machinery for synthesizing their own proteins. Several phage-specific early proteins were reported to modify/inactivate indispensable bacterial proteins in order to exploit them preferentially for phage development. Inhibition of indispensable proteins, associated mainly with bacterial transcription/translation, replication and cell division, in turn leads to cell death eventually (1). The lethal interaction between above proteins, though promising for novel antibacterial drug discovery, was only exploited to screen some antistaphylococcal compounds lately (2).

Mycobacteriophages such as L5 (3), Bxb1 (3), and L1 (4) were studied elaborately at the molecular level during last ~20 years. Several gene regulatory elements such as promoters, operators, *att*P sites, integrase, excisionase, repressor, etc. were cloned from the above mycobacteriophages and characterized at length. Interestingly restriction map, repressor, operator and an early promoter of L1 were found to be 100% identical to those of L5. Phage L5 was reported to inhibit protein synthesis in *M. smegmatis* immediately after infection (3) and its two genes, *gp63* and *gp64* were hypothesized to be toxic to *M. smegmatis* (3). Little is known at present about the above two L5 genes and the L5 gene(s) that inhibits protein synthesis in host.

Our preliminary data suggest that L1 also carries an ortholog of L5 *gp64* gene and inhibits protein synthesis in *M. smegmatis*. Here we show that overexpression of L1 *gp64* (designated *hlg1*: host lethal gene 1) retards the growth of both *M. smegmatis* and *E. coli* cells significantly and the protein product of *hlg1* (termed HLG1) is involved in its growth retardation activity. HLG1, a putative delayed early lipoprotein, contributes little to vegetative growth of L1 and inhibits nucleic acid and protein biosynthesis in both *M. smegmatis* and *E. coli* severely.

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RESULTS AND DISCUSSION

Overexpression of *hlg1* retards growth of *M. smegmatis*

To prove the cytotoxicity of *hlg1* conclusively, the vector p1100, constructed by cloning *hlg1* at the downstream of acetamide inducible promoter in pJAM2, was transformed to *M. smegmatis* mc^{2}155 and the growth of a resulting transformant, *M. smegmatis* (p1100), was studied in presence/absence of acetamide. *M. smegmatis* (p1100) almost did not grow on acetamide containing hard agar even after ~3 days of incubation but the growth of *M. smegmatis* (pJAM2) was only marginally affected under identical condition (Fig. 1A). Interestingly, *M. smegmatis* expressing *hlg1* from a single copy plasmid p1117 grew well on hard agar supplemented with acetamide. Contrary to above, growth of *M. smegmatis* (p1100) and *M. smegmatis* (pJAM2) in liquid medium were reduced about 42% and 20%, respectively, when they were exposed to acetamide for ~20-24 h (Fig. 1B). This diminished growth retardation of *M. smegmatis* might be due to the lesser period of expression of *hlg1* by acetamide in liquid broth. The growth, however, resumed again when acetamide was withdrawn from liquid culture (data not shown). Further study revealed that cell size of above acetamide-treated *M. smegmatis* (p1100) cells was about 50% of that of acetamide-untreated cells (Fig. 1C). The reduced size of *M. smegmatis* (p1100) cell might account for its reduced cell density in liquid medium. It was noticed that leaky

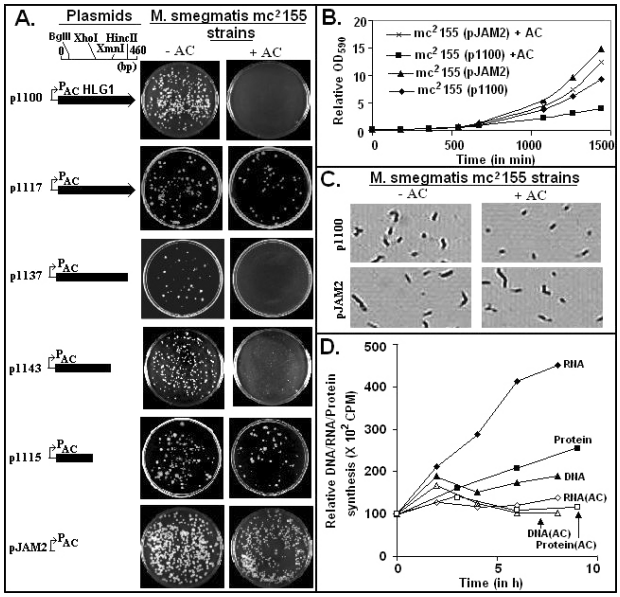

Fig. 1. Effect of HLG1 on *M. smegmatis*. (A) Growth of different *M. smegmatis* mc^{2}155 strains on hard agar. Culture aliquots were spread on Middlebrook 7H9 kanamycin hard agar supplemented with/without 0.5% acetamide (AC) and grown for 3-5 days at 37^{o}C. Each plate picture pair (row wise) represents the growth status of a particular *M. smegmatis* strain (carrying distinct pJAM2 derivative) both in presence and absence of AC. Each pJAM2 derivative (shown on the left side of respective picture) carries specific L1 DNA under AC inducible promoter (P_{AC}) and encodes either intact HLG1 (solid arrow) or truncated HLG1 (bar). A physical map of HLG1 is also given. *M. smegmatis* (pJAM2) was used as a control here. Each *M. smegmatis* strain was grown 3-6 times separately on hard agar and only the representative pictures are presented here. (B) Growth kinetics of *M. smegmatis* mc^{2}155 (p1100). A log phase culture of *M. smegmatis* (p1100), grown in Middlebrook 7H9 kanamycin broth, was split into two equal parts. At 0 min, one part was made 0.5% with AC and both parts were continued to grow at 37^{o}C. The OD_{590} values of the aliquots, withdrawn from both the cultures at different time intervals, were measured and plotted against the time of growth. At 0 min, the density of cell culture was considered as 1%. Growth of each strain in liquid broth was studied 3 times and one representative result is included here. Control- *M. smegmatis* (pJAM2). (C) Microscopic pictures of *M. smegmatis* mc^{2}155 (p1100). Cells grown in the above Middlebrook 7H9 kanamycin broth supplemented with/without AC were smeared on a glass slide, heat-fixed, stained with carbol fuchsin and their pictures were taken using a Nikon dark field microscope (Model TE3000, Nikon, Tokyo, Japan) at X 6000 magnification. Control- *M. smegmatis* (pJAM2). (D) Kinetics of incorporation of radioactive precursors into RNA, DNA, and proteins of *M. smegmatis* mc^{2}155 (p1100). Cell culture ($OD_{590} \approx 0.2$) was split into two equal parts. At 0 h, AC was added to one part to the final concentration of 0.5% and both parts were continued to grow at 37^{o}C. Aliquots, taken out at regular interval from both cultures, were pulsed with ^{3}H-Uracil or ^{35}S-Methionine separately, processed as described in Materials and Methods and amount of radioactivity incorporated into DNA/RNA or protein molecules of *M. smegmatis* (p1100), respectively, were plotted against the time of culture collection. Curves marked DNA/DNA (AC), RNA/RNA (AC), and Protein/Protein (AC) denote the status of incorporation of specific radioactive precursors into the DNA, RNA and protein molecules of both AC-uninduced/induced *M. smegmatis* (p1100) cells during the period of study. Counts present in 0 h samples were considered as 100%. Incorporation of the particular radioactive precursor to each culture was studied 3 times and one representative data is presented here.

expression of *hlg1* from p1100 also affected the growth of *M. smegmatis* in both media to a little extent. The results suggest that overexpression of *hlg1* somehow impairs *M. smegmatis* cells of reaching their full growth.

Region of *hlg1* retards cell growth

To map the region of *hlg1* responsible for growth retardation, some derivatives of p1100 were constructed which are capable of encoding different truncated HLG1 (a protein expressed by *hlg1*). As shown in Fig. 1A, *M. smegmatis* (p1137) expressing a truncated HLG1 (HLG1 without its 14 C-terminal amino acid residues) did not grow at all in presence of acetamide. Another truncated HLG1 (HLG1 without its 42 C-terminal amino acid residues) also diminished cell growth upon overexpression from p1143 but the extent of toxicity of this second deleted HLG1 is much less than that of either intact HLG1 or first truncated HLG1. Overexpression of third truncated HLG1 from p1115, which carries first 56 N-terminal amino acid residues only, did not cause any harm to cell growth (Fig. 1A). Additional study with p1132 (where a stop codon was inserted just at the upstream of initiation codon of HLG1) revealed that *hlg1* transcript did not retard growth of *M. smegmatis* (Fig. S1A). Taking together the data suggest that it is the protein product of *hlg1* which is responsible for growth retardation and secondly, 57-114 amino acid residues of HLG1 are absolutely necessary for its growth retardation activity. No domain or motif, however, was detected within the above region.

HLG1 drastically inhibits DNA, RNA, and protein synthesis in *M. smegmatis*

To see the effect of HLG1 more precisely, incorporations of ^{3}H-Uracil and ^{35}S-Methionine precursors into nucleic acid and protein molecules of acetamide induced *M. smegmatis* (p1110) cells were studied separately and the data were presented in Fig. 1D. The total cellular RNA synthesis in *M. smegmatis* (p1110) was found not to increase significantly throughout 9 h after induction of HLG1 synthesis. In contrast, cellular DNA or protein synthesis increased up to ~3 h under identical condition, thereafter, there was decrease in synthesis. After 6 h of induction, DNA and protein synthesis remained almost static. In uninduced *M. smegmatis* (p1100) and in induced/uninduced *M. smegmatis* (pJAM2), there were continuous synthesis of nucleic acid and protein as expected (Fig. 1D and data not shown). Taken together, the data suggest that primary target of HLG1 may be the total cellular transcription and decrease in cellular DNA and protein synthesis might have arisen due to inhibition of cellular RNA synthesis in induced *M. smegmatis* (p1110) cells. We speculate that growth retardation mediated by HLG1 might have arisen due to the blockage of transcription alone or it might be due to the additive effects of inhibition of all macromolecular biosynthesis associated directly or indirectly with transcription/translation.

HLG1 is lethal to *E. coli* too

To overexpress HLG1 in *E. coli* as an N-terminal his-tagged variant, a vector p1184, harboring HLG1 encoding gene under an IPTG (isopropyl-β-D-thiogalactopyranoside) inducible promoter, was utilized and analysis of different *E. coli* cell extracts showed an overexpressed protein of ~13 kDa only in induced *E. coli* (p1184) cell extract (Fig. 2A). It failed to bind to both Ni-NTA column and anti-his antibody. Surprisingly, another protein entity with the molecular mass of ~20 kDa, present only in the induced *E. coli* cell extract, reacted with anti-his antibody (Fig. 2A). The data suggest that the ~13 kDa protein might have arisen due to the cleavage of N-terminal end (including histidine tag) of full length (histidine-tagged) HLG1 and as a result, level of full length HLG1 dropped in *E. coli*. If the above hypothesis is true, then HLG1 might be toxic to *E. coli* too. It was indeed found that *E. coli* (p1184) cells did not grow either on hard agar (Fig. 2B) or in liquid media (Fig. 2C) in presence of IPTG. Growth of above cells was resumed when IPTG was withdrawn from media (data not shown). Interestingly, HLG1 looked more toxic to *E. coli* than that to *M. smegmatis* (comparing Fig. 1B with Fig. 2C). One of the reasons of increased toxicity might be the higher as well as longer period of expression of HLG1 in the former organism. Growth of *E. coli* (p1219) expressing a truncated HLG1 (HLG1 without its 42 C-terminal amino acid residues) was not retarded at all (Fig. 2B) though same protein showed a little toxicity to *M. smegmatis* (Fig. 1A).

Incorporation study with ^{3}H-Uracil showed that transcription in *E. coli* (p1184) cells was severely affected immediately after the initiation of HLG1 synthesis with IPTG (Fig. 2D). In contrast, DNA replication remains unaffected during first ~50 min and thereafter, it synthesis decreases significantly. Cellular protein synthesis in induced *E. coli* (p1184) decreased only after 50 min of initiation of HLG1 synthesis (Fig. 2D). The patterns of nucleic acid and protein biosynthesis in IPTG induced/uninduced *E. coli* (pQE31) were nearly similar to that of uninduced *E. coli* (p1184) (data not shown). The results together suggest that HLG1 inhibits key macromolecular biosynthesis in both *E. coli* and *M. smegmatis* possibly by similar mechanism.

Transcription of *hlg1* gene

To reveal the time of expression of *hlg1* during vegetative growth of L1, L1cI^{-} infected *M. smegmatis* culture aliquots were collected at various time points (0, 5, 15, 30, 60, and 90 min) and total RNA isolated from each sample was analyzed by RT-PCR. As shown in Fig. 3, *hlg1*-specific transcript appeared at 15 min post infection and its synthesis continues throughout the whole late period. RNA samples utilized for preparing cDNAs were found not to contain any L1 DNA (Fig. 3). Total RNA isolated from uninfected *M. smegmatis* also did not yield any *hlg1*-specific band. Additional study showed that L1 lysogen synthesizes L1 repressor-specific transcript but not the *hlg1*-specific transcript (Fig. 3). The latent period of L1 was

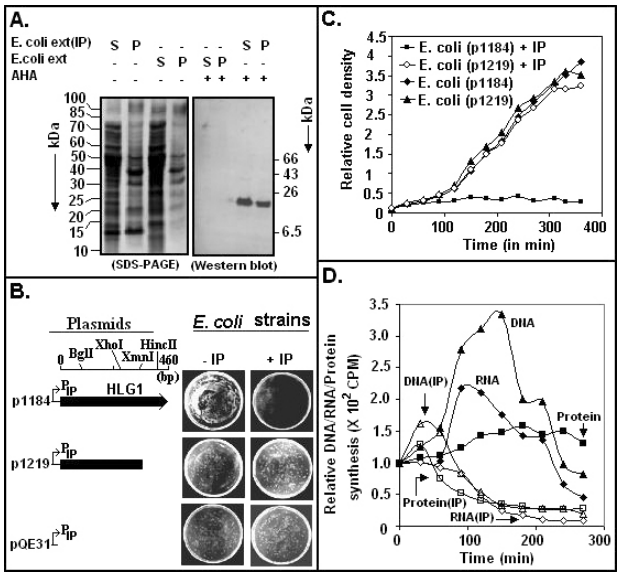

Fig. 2. Effect of HLG1 on *E. coli*. (A) Overexpression and detection of HLG1. The supernatant (S) and pellet (P) fractions, prepared from IPTG (IP) induced/uninduced *E. coli* (p1184) cells, were analyzed by SDS-12% PAGE. Nearly equal amount of protein fraction was loaded in each lane. Protein fractions resolved by SDS-PAGE (picture shown) were transferred to a nitrocellulose membrane and western blot analysis was performed by standard procedure using anti-his antibody (AHA). Sizes of the marker protein bands are also presented. (B) Growth of *E. coli* (p1184/p1219) strains on hard agar. Culture aliquots, spread on LB hard agar supplemented with kanamycin, ampicillin and 100 μM IPTG (IP) or no IP, were grown 16 h at 37°C. Each picture pair (row wise) shows the growth status of a particular *E. coli* (carrying distinct pQE31 derivative) in presence and absence of IP. L1 DNA fragments encoding intact/truncated HLG1 were cloned at the downstream of IP inducible promoter (P_{IP}) in pQE31. Each strain was grown 3-4 times on hard agar plates. Only representative pictures are given here. See Fig. 1A for other details. Control: *E. coli* (pQE31). (C) Growth kinetics of *E. coli* (p1184 or p1219). A log phase *E. coli* culture, grown in LB-kanamycin-ampicillin broth was split into two equal parts. At 0 min, one part was made 100 μM with IP and both parts were continued to grow at 37°C. The OD_{590} values of the aliquots, withdrawn from IP induced/uninduced cell culture at different time intervals, were measured and plotted against the time of growth. At 0 min, the density of cell culture was considered as 0.1%. Each experiment was performed 3 times and data of one representative experiment is included here. Control: *E. coli* (pQE31). (D) Kinetics of incorporation of radioactive precursors into RNA, DNA, and proteins of *E. coli* (p1184). Cell culture, grown in M9 broth (supplemented with kanamycin, ampicillin and 0.01% thiamine) up to $OD_{590} \approx 0.2$, was split into two equal parts. At 0 min, 500 μM IPTG was added to one part and both parts were continued to grow at 37°C. Aliquots, taken out at regular interval from both parts, were pulsed with specific radioactive precursor and processing of the aliquots were performed by the same way as described in Fig. 1D. Curves marked DNA/ DNA (IP), RNA/RNA (IP) and Protein/Protein (IP) indicate the status of specific radioactive precursors incorporated into the DNA, RNA and protein molecules of both IP-uninduced/induced *E. coli* (p1184) during the period of study. Counts present in 0 min samples were considered as 1. Incorporation of the particular radioactive precursor to each culture was studied 3 times and one representative data is presented here.

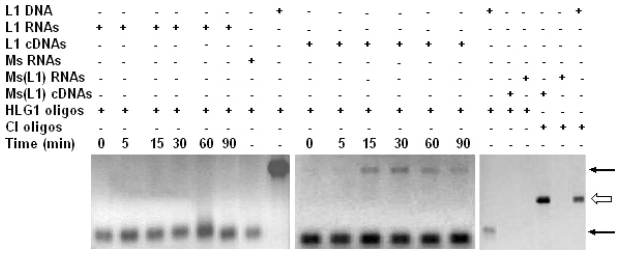

Fig. 3. Analysis of HLG1-transcripts. The cDNA/DNA molecules, prepared from RNA/DNA samples by RT-PCR/PCR, were analyzed by 1.5% agarose gel electrophoresis. Abbreviations: Ms RNA, *M. smegmatis*-specific RNA; HLG1 oligos, P64R and P64F; L1 RNAs, RNAs isolated from L1 infected *M. smegmatis*; L1 cDNA, cDNA prepared from L1 RNAs; L1 DNA, DNA isolated from L1c*lts*391 phages; Ms(L1) RNAs, RNAs isolated from L1c*lts*391 lysogen; Ms(L1)cDNAs, cDNAs prepared from Ms(L1)RNAs. Sequences of CI oligos were reported before (Ganguly et al., 2004). Solid and hollow arrows indicate HLG1-specific and cI gene-specific DNA fragments, respectively.

reported to be around 100 min (5). Therefore the data suggest that *hlg1* is a delayed early gene.

Inhibition of HLG1 expression has little effect on L1 growth

To see whether HLG1 is essential for lytic growth of L1, a plasmid p1203 was constructed which carried *hlg1* in antisense orientation under the control of a tetracycline inducible promoter P_{Tet}. In presence of tetracycline, *M. smegmatis* harboring p1203 would block the growth of infected L1 only if HLG1 is indispensable for L1 growth. To test this hypothesis, plating efficiency of L1cI$^-$ on *M. smegmatis* (p1203) was determined both in presence and absence of tetracycline (Fig. S1B). It was observed that plating efficiency as well as plaque diameter of L1 in presence of tetracycline is nearly identical to that in absence of tetracycline (Table S1). Plasmid p1235 harboring a promoterless *xylE* gene at the downstream of antisense *hlg1* in p1203 drove the expression of xylE in *M. smegmatis* indicating that transcription of antisense *hlg1* occurs positively within cell (Fig. S1C). No HLG1 homolog was detected either in L1 or in *M. smegmatis*. The data therefore suggest that HLG1 does not participate in L1 growth.

HLG1 is a putative lipoprotein

BLAST search (www.ncbi.nim.nih.gov) revealed that GP70, *gp64*, and GP120 encoded by mycobacteriophages D29, Che12, and Omega, respectively, are nearly 75-82% identical over the entire length of HLG1, whereas, several proteins from other phage (e.g., bIL309, Twort, PY54, etc.) showed 40-55% identity with HLG1. Further studies suggest that HLG1 harbors a signal peptide and a lipo-box motif around its 1-20 and 18-21 N-terminal amino acid residues, respectively. Its 21st residue, cysteine, is the putative target site for binding palmitoyl and/or diacylglycerol moiety. The data suggest that HLG1 acts as a lipoprotein. Unlike HLG1, other phage lipoproteins are involved in superinfection exclusion (6-8). Interestingly, HLG1 without its N-terminal 20 amino acid residues is also toxic to *E. coli* (Fig. S1D) confirming the role of C-terminal end of HLG1 in its lethality. Secondly, presence of signal peptidase cleavage site at the N-terminal end of HLG1 is most possibly responsible for further lowering its level in *E. coli* (Fig. 2A).

In summary, we report for the first time a mycobacteriophage-specific delayed early gene which when overexpressed is lethal not only to host *M. smegmatis* but also to *E. coli*. In depth characterization of this gene may pave the way to discover novel broad-spectrum antibacterial compounds which in turn may restrict bacterial infections caused by multiple antibiotic-resistant bacterial strains including *M. tuberculosis*.

MATERIALS AND METHODS

Bacteria and phage strains, plasmids and growth conditions

M. smegmatis mc^{2}155 and *E. coli* were routinely grown in Middlebrook 7H9 medium (5) and LB (Luria-Bertani) broth (9), respectively. Growth media were supplemented with appropriate antibiotic, 0.5% acetamide or 100 μM IPTG whenever needed. The wild-type mycobacteriophage L1, its mutants L1cI$^-$, L1c*lts*391, and phage assay procedure were described previously (5).

Plasmids pJAM2 (10), pBP10 (11) and pMIND (12) were procured from Dr. V. Nagaraja (IISc, India), Dr. K. G. Papavinasasundaram (NIMR, UK) and Dr. G. R. Stewart (Imperial College, UK), respectively. The vector pQE31, *E. coli* M15 (pREP4) (Qiagen, Germany) and plasmid pET28a (Novagen, USA) were obtained from late Dr. P. Roy (Bose Institute, India). *E. coli* M15 (pREP4) was mentioned simply as *E. coli* here.

DNA/RNA isolations and manipulations

Genomic DNA was isolated from the wild-type L1 phage according to the standard procedure (5). RNAs were isolated from L1cI$^-$ infected *M. smegmatis* or *M. smegmatis* (L1c*lts*391) by a standard method (3). Plasmid DNA isolation, DNA digestion by restriction enzymes, modification of DNA fragments, DNA ligation, competent cell preparation, transformation, agarose and polyacrylamide gel electrophoresis, polymerase chain reaction (PCR), reverse transcriptase polymerase chain reaction (RT-PCR) for amplification of cDNA molecules, DNA sequencing, western blotting etc. were performed according to standard procedures (4, 9, 13). All PCR amplified DNA fragments were confirmed by DNA sequencing.

Plasmid construction

Plasmid p1095 was constructed by cloning an L1 DNA region [amplified from L1 genomic DNA by Taq polymerase using primers P64F (5′-GAGAGATCTTAGTGCAG ATCAACGACTTCG) and P64R (5′-GAGTCTAGATCAGCGCAGTTCGATGTC)] into a TA cloning vector (Genei, India). Sequence of L1 DNA insert of p1095 was found 100% identical to that of an L5 region carrying *gp64*. To construct p1100, a *Bgl*II-*Pvu*II fragment of p1095 harboring L1 *gp64* (*hlg1*) was subcloned into *Bam*HI

and *Sca*I sites of pJAM2 (10).

To form p1117, a ~2 kbp *Hind*III-*Xba*I DNA fragment of p1100 was cloned into *Hind*III and *Spe*I sites of p1097 [constructed by blocking the *Hind*III site (located at 4035 bp) of pBP10].

To construct p1137, a 407 bp *Bgl*II-*Hinc*II fragment of p1095 was subcloned into *Bam*HI and *Sca*I sites of pJAM2.

Plasmid p1143 was constructed by ligating a 325 bp *Bgl*II-*Xmn*I fragment of p1095 with 9.4 kb *Bam*HI-*Sca*I fragment of pJAM2.

To construct p1108, unique *Xho*I site (present within *hlg1*) of p1095 was treated with Klenow polymerase and T4 ligase successively. A *Bgl*II-*Pvu*II fragment of p1108 harboring mutated *hlg1* was subcloned into *Bam*HI and *Sca*I sites of pJAM2 to form p1115.

To construct p1203, blunt ended *Nco*I fragment of p1095 was cloned into *Eco*RV site of pMIND. To generate p1215, a promoterless *xylE* reporter gene [amplified from pLL38 DNA (14) by ProofStart Polymerase using suitable primers] was cloned into the blunt ended *Mlu*I site of p1203.

Plasmid p1104 was constructed by cloning an L1 DNA insert [amplified from L1 genomic DNA by ProofStart Polymerase using primers Pr64f (5′-GATATCACATGAA GAAGATCATCGC) and Pr64r (5′-AAGCTTTCAGCGCAGTTCGATGTC)] into *Mlu*NI site of pCAP^{s} (Roche Applied Science, Germany). To generate p1184, an *Eco*RV-*Hind*III fragment of p1104 containing L1 DNA was subcloned into *Sma*I and *Hind*III double digested pQE31. To create p1219, an *Eco*RV-*Xmn*I fragment from p1104 was cloned into *Sma*I site of pQE31.

Overexpression of HLG1 in *E. coli*

A log phase *E. coli* (p1184) culture, grown in LB broth (supplemented with kanamycin and ampicillin) was induced with 100 μM IPTG for 4 h at 23°C. Cells harvested by centrifugation were resuspended in 1/20 volume of lysis buffer [20 mM Tris-Cl (pH 8.0), 500 mM NaCl, 5% glycerol, 1% EDTA and 100 μg/ml PMSF]. The sonicated cell extract was centrifuged at 12000 rpm for 1 h at 4°C and both the supernatant and pellet fractions were collected for analysis.

Incorporation of radioactive precursors

Incorporation of ^{3}H-Uracil into the nucleic acid of bacteria was studied by a modified form of an earlier method (15). Briefly, cell aliquot withdrawn from growing bacterial culture at each time point was pulsed with 5 μCi ^{3}H-Uracil (specific activity 8 Ci/mmol) for 4 min. The pulsed aliquot was split into two equal parts and both parts were treated with 10% TCA (ice-cold). TCA precipitate was collected from one part, washed successively with 1 N HCl, cold water, and 95% ethanol and radioactivity retained on it was measured by a liquid scintillation counter. The counts represent the amount of radioactivity incorporated into DNA and RNA at a particular time of study. The remaining part was treated with 3 N KOH for 24 h at 37°C followed by treatment of TCA precipitate by identical manner as described above. The counts present in alkali-treated sample denote the amount of radioactive precursor incorporated into DNA alone. The amount of radioactive precursor incorporated into RNA was determined by deducting the counts of KOH-treated sample from that of KOH-untreated sample.

Incorporation of ^{35}S-Met into bacterial proteins was studied by a modified form of earlier procedure (3). Briefly, cell aliquot collected from growing bacterial culture at regular interval was pulsed with 5 μCi ^{35}S-Met (specific activity 1000 Ci/mmole) for 4 min followed by lysing the cells with 500 μl 10% TCA (ice cold). Radioactivity count present in TCA precipitate was measured as described above.

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※ Additional files for supplementary materials, Fig. S1 and Table S1, Click http://bmbreports.org

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