Role of DLP12 lysis genes in *Escherichia coli* biofilm formation

Faustino A. Toba,^{1†} Mitchell G. Thompson,¹ Bryan R. Campbell,^{1†} Lauren M. Junker,^{1‡} Karl-Gustav Rueggeberg¹ and Anthony G. Hay^{1,2}

Correspondence
Anthony G. Hay
agh5@cornell.edu

¹Department of Microbiology, Cornell University, Ithaca, NY 14853, USA

²Institute for Comparative and Environmental Toxicology, Cornell University, Ithaca, NY 14853, USA

Phages have recently been implicated as important in biofilm development, although the mechanisms whereby phages impact biofilms remain unclear. One defective lambdoid phage carried by *Escherichia coli* K-12 is DLP12. Among the genes found in DLP12 are *essD*, *ybcS* and *rzpD/rzoD*, which are homologues of the Lambda phage genes encoding cell-lysis proteins (S, R and Rz/Rz¹). The role that these DLP12 lysis genes play in biofilm formation was examined in deletion mutants of *E. coli* PHL628, a curli-overproducing, biofilm-forming K-12 derivative. Strains lacking *essD*, *ybcS* and *rzpD/rzoD* were unable to form wild-type biofilms. While all mutants were compromised in attachment to abiotic surfaces and aggregated less well than the wild-type, the effect of the *essD* knockout on biofilm formation was less dramatic than that of deleting *ybcS* or *rzpD/rzoD*. These results were consistent with electron micrographs of the mutants, which showed a decreased number of curli fibres on cell surfaces. Also consistent with this finding, we observed that expression from the promoter of *csgB*, which encodes the curli subunits, was downregulated in the mutants. As curli production is transcriptionally downregulated in response to cell wall stress, we challenged the mutants with SDS and found them to be more sensitive to the detergent than the wild-type. We also examined the release of ¹⁴C-labelled peptidoglycan from the mutants and found that they did not lose labelled peptidoglycan to the same extent as the wild-type. Given that curli production is known to be suppressed by *N*-acetylglucosamine 6-phosphate (NAG-6P), a metabolite produced during peptidoglycan recycling, we deleted *nagK*, the *N*-acetylglucosamine kinase gene, from the lysis mutants and found that this restored curli production. This suggested that deletion of the lysis genes affected cell wall status, which was transduced to the curli operon by NAG-6P via an as yet unknown mechanism. These observations provide evidence that the S, R and Rz/Rz¹ gene homologues encoded by DLP12 are not merely genetic junk, but rather play an important, though undefined, role in cell wall maintenance.

Received 18 September 2010

Revised 26 February 2011

Accepted 14 March 2011

INTRODUCTION

Recently, phages and phage remnants have been investigated for their role in numerous aspects of host cell physiology, including biofilm development (Wang *et al.*, 2010). Prophage genes have been found to be upregulated in biofilm cells from several genera, yet the exact role that they play is unclear (Stanley & Lazazzera, 2004; Wang *et al.*, 2010; Whiteley *et al.*, 2001). Phage exposure and prophage activity have been implicated in biofilm dispersion,

enhanced biofilm traits such as antibiotic resistance, attachment and phage tolerance, and in metabolite recycling (Wang *et al.*, 2010; Webb *et al.*, 2003, 2004). Work by Rice & Bayles has also shown that a phage-like holin *cidA* is important for lytic release of DNA, the latter being necessary for normal biofilm formation by *Staphylococcus* (Bayles, 2007). Wang *et al.* (2010) have recently shown that in addition to biofilm formation, cryptic phages have an important impact on resistance to a number of stresses, including antibiotic exposure, while also having a significant impact on growth.

Nearly 70 % of all sequenced bacterial chromosomes contain defective prophages, with some chromosomes being composed of up to 20 % bacteriophage genetic material (Casjens, 2003; Wang *et al.*, 2010). *Escherichia coli* MG1655 contains a number of defective lambdoid phage remnants, including DLP12 (defective Lambda prophage),

[†]Present address: Medical Instill Technologies, Inc., 201 Housatonic Ave., New Milford, CT 06776, USA.

[‡]Present address: Johnson and Johnson Consumer Products Worldwide, 185 Tabor Rd, Morris Plains, NJ 07950, USA.

Abbreviations: AMB, ammonium molybdate; CSLM, confocal scanning laser microscopy; CV, crystal violet; NAG-6P, *N*-acetylglucosamine 6-phosphate; PVC, polyvinyl chloride.

which is found at the 12th minute of the chromosome (Campbell, 1994; Lindsey *et al.*, 1989). In DLP12, four ORFs have been identified, *essD*, *ybcS*, *rzpD* and *rzoD*, which are homologous to the genes encoding the lytic functions of lambdoid phage P21 (*s*, *r*, *r_z* and *r_z¹*, respectively) (Campbell, 1994; Lindsey *et al.*, 1989). Although studies have shown that other phages can contribute to host fitness (Wang *et al.*, 2010; Campbell, 1994; Lindsey *et al.*, 1989), the role of the DLP12 lysis genes in *E. coli* has only recently received attention (García-Contreras *et al.*, 2008; Wang *et al.*, 2009, 2010). In the canonical lambdoid virus, S is a holin through which the endolysin (R) and spannin (Rz/Rz¹) escape into the periplasmic space. R and Rz/Rz¹ are then able to degrade the cell wall and destabilize the outer membrane, causing host cell lysis (Campbell, 1994; Wang *et al.*, 2009; Young *et al.*, 2000; Zhang & Young, 1999). More recently, another lysis paradigm has been described in which some R homologues are exported to the periplasm in a sec-dependent manner (Xu *et al.*, 2005). There they are tethered to the outside of the cytoplasmic membrane until the membrane is perturbed by the so called ‘pinholin’ (S). This event releases and activates R, causing host cell lysis (Xu *et al.*, 2005).

Given their lytic function, one might assume the retention of these DLP12 homologues by *E. coli* to be an evolutionary accident, that these lysis genes are just genetic interlopers that either are not expressed or are in a state of mutational decay and encode non-functional proteins (reviewed by Casjens, 2003). However, a genome-wide expression study has shown that the DLP12 lysis genes are actively transcribed in a σ^E -dependent manner (Rhodius *et al.*, 2006). Another study has also shown that the product of *ybcS* is an active endolysin capable of degrading Gram-positive and Gram-negative peptidoglycan (Srividhya & Krishnaswamy, 2007), although its physiological relevance in *E. coli* was not examined.

Recent work has also shown that the DLP12 lysis gene products are upregulated by the small transcriptional regulator Hha in *E. coli* BW25113. This small regulatory protein suppresses biofilm formation in this strain by repressing expression of the genes encoding type I fimbriae (García-Contreras *et al.*, 2008). Additionally, although Wang and co-workers reported that the deletion of any of the DLP12 lysis genes enhanced biofilm formation by BW25113 (Wang *et al.*, 2009), they have more recently demonstrated that loss of the entire DLP12 prophage reduces biofilm formation by that strain (Wang *et al.*, 2010).

Given that different *E. coli* strains are known to employ distinct biofilm formation strategies that rely on different surface structures, such as flagella, fimbriae, extracellular polymeric substances and/or curli (Barnhart & Chapman, 2006; Barnhart *et al.*, 2006; Pratt & Kolter, 1999; Prigent-Combaret *et al.*, 2000), we chose to examine the role of the DLP12 lysis genes in biofilm formation by *E. coli* PHL628. This strain relies on curli rather than type 1 fimbriae for

biofilm formation and is an MG1655 (F[−]) derivative that harbours an *ompR234* mutation (Vidal *et al.*, 1998). This *ompR* allele has been found in clinical isolates and stimulates enhanced expression of the *csgBAC* operon, with a concomitant increase in curli production (Jubelin *et al.*, 2005; Ryu *et al.*, 2004; Stanley & Lazazzera, 2004; Vidal *et al.*, 1998). Curli are important in pathogenesis, are recognized as a pathogen-associated molecular pattern, and have been shown to induce bacterial engulfment by eukaryotic cells (reviewed by Barnhart *et al.*, 2006).

In this work we report the effect of individual deletions of each of the DLP12 lysis genes (Δ *essD*, Δ *ybcS* and Δ *rzpD/rzoD*) on aggregation, attachment and biofilm architecture in *E. coli* PHL628. Transcription from a *P_{csgB}*-GFP promoter fusion was monitored and the production of curli was examined microscopically. Given the putative role of the lysis genes in cell wall metabolism, we also monitored the status of the cell wall and showed that deletion of *nagK* was enough to restore curli expression in the DLP12 lysis mutants. We report here on these results, which implicate the products of the DLP12 lysis genes in cell wall maintenance and indirectly in biofilm development by curli-producing *E. coli*.

METHODS

Bacterial strains and growth conditions. *E. coli* strain PHL628 is an MG1655 derivative with an *ompR234* mutation (Vidal *et al.*, 1998). *E. coli* PHL628 and mutants were routinely grown on Luria-Bertani (LB) medium at 37 °C overnight with shaking (150 r.p.m.) and supplemented with 50 µg kanamycin (Kan) ml^{−1}, unless otherwise stated (Table 1). To obtain biofilms, *E. coli* PHL628 and mutants were grown on minimal medium (MSM) (McCullar *et al.*, 1994) supplemented with 0.2 % (w/v) casamino acids (CA) and Kan, unless otherwise stated. When required, plates and media were supplemented with 150 µg ampicillin (Amp) ml^{−1} and/or 25 µg chloramphenicol (Cm) ml^{−1}. All chemical compounds and reagents were obtained from Fisher Scientific.

Mutants. Deletion mutants were constructed by allele replacement using the Lambda RED strategy, as described elsewhere, and using the primers described in Table 1 (Choi & Schweizer, 2005). Briefly, Cm-interrupted versions of the genes were created using PCR-mediated ligation (Datsenko & Wanner, 2000). In brief, each construct consisted of 5′ and 3′ end 500 bp fragments homologous to the gene of interest linked by a 1 kb Cm-resistance cassette flanked with flip recombinase target (FRT) sites. This linear DNA was transformed into wild-type *E. coli* PHL628 cells expressing the Lambda RED recombinase. The 5′ and 3′ end homology regions allowed the RED system to replace the wild-type allele with an interrupted mutant version. Mutant selection was carried out on plates supplemented with the appropriate antibiotic. After recombination had been confirmed, the Cm marker was removed using the FLP recombinase enzyme activity encoded on plasmid pCP20 (Datsenko & Wanner, 2000). All plasmid vectors were temperature-sensitive and were cured from the cells by growth at 43 °C. DLP12 Δ *nagK* double mutants were created using P1 phage transduction of Δ *nagK*:Cm from BW25113 Δ *nagK*:Cm into each DLP12 lysis gene mutant. The Cm marker was removed using the FLP recombinase procedure described above. The markerless knockout mutants were confirmed by PCR and sequencing (Cornell University Life Sciences Core Laboratories Center). PCR

Table 1. Strains, plasmids and primers used in this study

Primer, plasmid or strain	Sequence	Source or reference
Primers		
S ^{DLP12} Forward1	5'-GGGCCCCACTTCGTACACAGCTTAAACCCG-3'	This study
S ^{DLP12} Reverse1	5'-GGATCCTGGCGACAGTGGCAAGGTCTTTAT-3'	This study
R ^{DLP12} Forward1	5'-GGGCCCTGCGAGAGGTGAATAATGCCTCCA-3'	This study
R ^{DLP12} Reverse1	5'-GGATCCTTCGCTAGCTTCAGTTCTCTGGCA-3'	This study
Rz ^{DLP12} Forward1	5'-GGGCCCCACTTCGTGAGATTGAGCGTGAA-3'	This study
Rz ^{DLP12} Reverse1	5'-GGATCCGGAAGCGCGTGTGTATTGCTCAAA-3'	This study
essDp Forward1	5'-GGTACCTGCAGTACAGCAGAAATTGCCAC-3'	This study
essDp Reverse1	5'-TCTAGAGCGACACCCGTTGTTAACTTATCC-3'	This study
S ^{DLP12} internal Reverse	5'-GCGACACCCGTTGTTAACTTATCC-3'	This study
S ^{DLP12} internal Forward	5'-TGCGAGAGGTGAATAATGCCTCCA-3'	This study
R ^{DLP12} internal Reverse	5'-ACACCTTCCAGACCATCGTTACCA-3'	This study
R ^{DLP12} internal Forward	5'-ACTCGTCGTGAGATTGAGCGTGAA-3'	This study
Rz ^{DLP12} internal Reverse	5'-ATGACAGGCTGACGATGATGCAGA-3'	This study
Rz ^{DLP12} internal Forward	5'-AGCAGTGCAGATAGAGCTGACCAT-3'	This study
P1S	5'-GGATAAGTTAACAACGGGTGTCGCGTGTAGGCTGGAGCTGCTTCG-3'	This study
P2S	5'-TGGAGGCATTATTCACCTCTCGCACATATGAATATCCTCCTTA-3'	This study
P1R	5'-TGGTAACGATGGTCTGGAAGGTGTGTGTAGGCTGGAGCTGCTTCG-3'	This study
P2R	5'-TTCACGCTCAATCTCACGACGAGTCATATGAATATCCTCCTTA-3'	This study
P1Rz	5'-TCTGCATCATCGTCAGCCTGTCATGTGTAGGCTGGAGCTGCTTCG-3'	This study
P2Rz	5'-ATGGTCAGCTCTATCTGCACTGCTCATATGAATATCCTCCTTA-3'	This study
csgB promoter F- <i>KpnI</i>	5'-GGGGTACCCCAAAGACGAGGTTTGTACACGGCTTG-3'	This study
csgB promoter R- <i>XbaI</i>	5'-GCTCTAGAGCTTTCATGTTGTACACCTGGACCTG-3'	This study
Plasmids		
pBBR1MCS		Kovach <i>et al.</i> (1995)
pBBR1MCS:S ^{DLP12}		This study
pBBR1MCS:R ^{DLP12}		This study
pBBR1MCS:Rz ^{DLP12}		This study
pJBA110		Andersen <i>et al.</i> (1998)
pJBA110:P _{csgB}		Junker (2004)
pCP20		Datsenko & Wanner (2000)
pKD3		Datsenko & Wanner (2000)
pKD46		Datsenko & Wanner (2000)
Strains		
<i>E. coli</i> PHL628		Vidal <i>et al.</i> (1998)
<i>E. coli</i> PHL628 ΔS ^{DLP12}		This study
<i>E. coli</i> PHL628 ΔR ^{DLP12}		This study
<i>E. coli</i> PHL628 ΔRz ^{DLP12}		This study
<i>E. coli</i> PHL628 ΔS ^{DLP12}		This study
pBBR1MCS:S ^{DLP12}		
<i>E. coli</i> PHL628 ΔR ^{DLP12}		This study
pBBR1MCS:R ^{DLP12}		
<i>E. coli</i> PHL628 ΔRz ^{DLP12}		This study
pBBR1MCS:Rz ^{DLP12}		
<i>E. coli</i> PHL628 pJBA110:P _{csgB}		This study
<i>E. coli</i> PHL628 ΔS ^{DLP12}		This study
pJBA110:P _{csgB}		
<i>E. coli</i> PHL628 ΔR ^{DLP12}		This study
pJBA110:P _{csgB}		
<i>E. coli</i> PHL628 ΔRz ^{DLP12}		This study
pJBA110:P _{csgB}		

Table 1. cont.

Primer, plasmid or strain	Sequence	Source or reference
<i>E. coli</i> PHL628 ΔS^{DLP12} pBBR1MCS:S ^{DLP12} pJBA110:P _{csgB}		This study
<i>E. coli</i> PHL628 ΔR^{DLP12} pBBR1MCS:R ^{DLP12} pJBA110:P _{csgB}		This study
<i>E. coli</i> PHL628 ΔRZ^{DLP12} pBBR1MCS:RZ ^{DLP12} pJBA110:P _{csgB}		This study
<i>E. coli</i> PHL 628 $\Delta nagK$: <i>Cm</i>		This study
<i>E. coli</i> PHL628 ΔS^{DLP12} $\Delta nagK$ pJBA110:P _{csgB}		This study
<i>E. coli</i> PHL628 ΔR^{DLP12} $\Delta nagK$ pJBA110:P _{csgB}		This study
<i>E. coli</i> PHL628 ΔRZ^{DLP12} $\Delta nagK$ pJBA110:P _{csgB}		This study

primers were supplied by IDT and amplifications were done on a PTC-200 DNA Engine thermal cycler (MJ Research).

Complements of the lysis genes were constructed using full wild-type versions of the genes cloned downstream of the *lac* promoter of pBBR1MCS-1 (Table 1) (Kovach *et al.*, 1995).

Reporter construct. To examine the expression from the *csgB* promoter, a fusion was created with the multi-copy vector pJBA110 containing the gene for a short-lived Gfp(LAA) (Junker, 2004). The *csgB* promoter was PCR-amplified with a primer set equipped with restriction endonuclease recognition sequences for *Xba*I and *Kpn*I. The resulting PCR product was cloned into pGEM-T Easy and electroporated into *E. coli* strain JM109. The clones were subsequently screened using blue-white selection on LB Amp plates and by PCR using primers T7F and M13R. The pGEM-T Easy:P_{csgB} plasmid and the pJBA110 vector were both digested with *Xba*I and *Kpn*I and gel-purified. The promoter fragments and the pJBA110 vector backbone were ligated together using T4 DNA ligase. The ligation reaction was then electroporated into *E. coli* PHL628. The resulting clones containing pJBA110:P_{csgB} were selected on LB (low salt, 5 g NaCl l⁻¹) supplemented with Kan and Amp, and were confirmed by PCR using primers for *gfp*, P(*gfp*)-upF, P(*gfp*)-upR and P(*gfp*)-downR, in combination with the promoter-specific primers.

Biofilm architecture. Biofilms were grown on Cultureware glass-bottomed dishes (MatTek) for imaging. Overnight cultures were inoculated (1%, v/v) into 3 ml MSM supplemented with CA. Dishes were incubated at 30 °C for 72 h with orbital shaking at 50 r.p.m. Medium was exchanged every 24 h. After 72 h, biofilms were stained with 0.025% (w/v) acridine orange and visualized under a TCS SP2 confocal microscope (Leica). To assess biofilm formation and architectural features, a total of eight confocal scanning laser microscopy (CSLM) z-image stacks (0.25 µm steps) were obtained for each mutant and then analysed with COMSTAT (Heydorn *et al.*, 2000). 3D renderings of the biofilms were generated from the image stacks using Leica/Leica lite software.

Attachment. Attachment to polyvinyl chloride (PVC) surfaces was studied as described by Genevax *et al.* (1996). Briefly, overnight cultures were diluted 1:5 to a total volume of 120 µl in 96-well PVC plates and incubated at 30 °C with shaking (50 r.p.m. on an orbital shaker) for 16 h. Turbidity (OD₆₀₀) was then measured with a

µQuant spectrophotometer (Bio-Tek), and 100 µl of 1% (w/v) crystal violet (CV) was added to each well. After 15 min incubation at room temperature, wells were thoroughly washed with water. Plates were allowed to dry for 15 min at room temperature. After drying, 100 µl 95% ethanol was added to each well and incubated for 10 min at room temperature. Next, CV absorbance (A₅₉₅) was measured in each well. The original well turbidity (OD₆₀₀) was compared with the CV absorbance (A₅₉₅). Experiments were conducted in triplicate.

Autoaggregation. Cell autoaggregation was measured as described by Barrios *et al.* (2006). Briefly, 4 ml of overnight low-salt LB culture of each knockout mutant and each complement was incubated statically for 12 h at 37 °C. OD₆₀₀ was measured from samples taken from the top 5 mm of each culture with minimal disturbance. Then, cultures were vortexed and sampled again. OD₆₀₀ values (undisturbed versus vortexed) for each mutant were compared as a measure of cell autoaggregation. Experiments were done in quadruplicate. To determine whether fimbriae played a role in autoaggregation, 1% (w/v) mannose (α-methyl-D-mannopyranoside) was added to the medium in a separate experiment that was carried out as described above.

Electron microscopy. Cells were grown on MSM CA at 37 °C with shaking overnight. Formvar copper grids (300 mesh, EMS) were floated on 25 µl of the overnight cultures for 1 min. Then the grids were transferred to 3% (w/v) ammonium molybdate (AMB) at pH 7.0 or 1% (w/v) potassium phosphotungstic acid (KPTA) for 2 min. Grids were then rinsed in MilliQ water (Millipore) for 1 min and allowed to dry before observation. Cells were studied on a Philips electron microscope at different magnifications ranging from ×7000 to ×45 000. Image snapshots were taken using a MicroFire digital camera and Optronics software.

Quantitative Congo red binding assay. Cells were plated on low-salt LB in order to obtain sufficient biomass and incubated at 30 °C for 72 h (Gophna *et al.*, 2001). After 3 days, cells were scraped from the plates and resuspended in 500 µl phosphate buffer (pH 7.0). Serial dilutions were prepared in phosphate buffer and the turbidity of each dilution was measured (OD₆₀₀). Then, cells were centrifuged (14 000 g, 3 min) and resuspended in 1 ml 0.002% (w/v) Congo red. Tubes were incubated at room temperature for 10 min to allow Congo red binding. Following incubation, tubes were centrifuged (14 000 g, 10 min), and the Congo red remaining in the supernatant

was measured (A_{500}) and compared with that of cell-free Congo red tubes that were treated as controls. Dilution turbidity (OD_{600}) and Congo red absorbance (A_{500}) were then plotted against one another. Experiments were done in triplicate.

Promoter fusion studies. Reporter plasmid pJBA110 carrying the *csgB* curli promoter (P_{csgB}) was transformed into each of the PHL628 strains (Junker, 2004). Cells were allowed to grow in MSM CA. At various time points fluorescence was measured using a Synergy HT spectrophotometer (Bio-Tek) with a 485/20 excitation filter and a 530/25 emission filter and corrected against the OD_{600} of the corresponding culture. Measurements were done in quadruplicate.

^{14}C -Labelled peptidoglycan release. The release of radiolabelled peptidoglycan degradation products to the supernatant was measured using a modification of a published method (Dubrac *et al.*, 2007). Briefly, overnight LB cultures were diluted 1:100 into MSM CA plus ^{14}C -labelled *N*-acetylglucosamine (NAG) ($0.2\ \mu\text{Ci}$, $7.4 \times 10^9\ \text{Bq}$, Perkin Elmer) and unlabelled NAG (0.05%) (MCNAG medium). To allow peptidoglycan labelling, cell cultures were grown at $30\ ^\circ\text{C}$ for 12 h. Then, labelled cells were washed three times with fresh MCNAG, and diluted 1:4 into MCNAG without radiolabel. Cells were then incubated for an additional 15 h. Periodically, samples were taken and boiled in 4% (w/v) SDS, then filtered through $0.2\ \mu\text{m}$ pore-size Durapore filters (Millipore). Filters were washed once with 0.1 M LiCl buffer and three times with MilliQ water. Then, the filters were placed in ScintiSafe Econo 1 scintillation cocktail (Fisher Scientific) and read in an LS 6500 scintillation counter (Beckman Coulter).

Membrane susceptibility to SDS. Cells were challenged with an inhibitory concentration of SDS to study their membrane susceptibility (Ize *et al.*, 2003). Overnight LB cultures were diluted 1:100 (OD_{600} 0.05) and exposed to 5% SDS, then incubated with shaking for 3 h at $37\ ^\circ\text{C}$,

after which OD_{600} was measured. The optical density was compared with that of unchallenged cultures incubated under the same conditions.

Statistical analyses The statistical significance of the differences between treatments was determined by calculating the *P* values derived from a two-tailed Student's *t* test using Microsoft Excel.

RESULTS

Biofilm changes

Biofilms of the DLP12 mutants (ΔessD , ΔybcS and $\Delta\text{rzpD/rzoD}$, henceforth referred to as Δs , Δr and $\Delta\text{rz/rz}^1$, respectively), were allowed to develop with the goal of studying the role of these genes in *E. coli* biofilm formation. The 3D renderings of the biofilm images obtained by confocal microscopy revealed that while the wild-type biofilm was characterized by numerous large, robust, tower-like structures, the mutant biofilms were largely flat and undifferentiated (Fig. 1, Table 2). We also observed that the mutant biofilms which did form detached readily from the substratum (data not shown). Even though we observed these differences in biofilm formation by the mutants, their planktonic growth was indistinguishable from that of the wild-type (data not shown).

Quantitative assessment of these biofilm images using COMSTAT software (Heydorn *et al.*, 2000) revealed that all the mutants were significantly compromised ($P \leq 0.05$) in total biomass (Fig. 1) and mean thickness (Fig. 1, Table 2).

Fig. 1. Deletion of the DLP12 lysis genes negatively affects PHL628 biofilm architecture. 3D biofilm renderings from CSLM *z*-stacks for (a) wild-type PHL628, (b) ΔS mutant, (c) ΔR mutant and (d) $\Delta\text{Rz/Rz}^1$ mutant. The COMSTAT image analysis values for mean $\pm$ SD biomass and thickness in Table 2 show that deletion of the DLP12 lysis genes (S, R, Rz/Rz¹) negatively affected biofilm biomass and thickness when compared with the wild-type PHL628 and the respective complements.

Table 2. COMSTAT image analysis values for the images shown in Fig. 1

Values shown are mean $\pm$ SD. WT, Wild-type; Δ , mutant; Comp., complemented strain.

	WT	S		R		$\Delta R_z/R_z^1$	
		Δ	Comp.	Δ	Comp.	Δ	Comp.
Biomass ($\mu\text{m}^3 \mu\text{m}^{-2}$)	7.160 $\pm$ 0.301	2.160 $\pm$ 0.365	6.306 $\pm$ 1.126	0.449 $\pm$ 0.033	4.679 $\pm$ 0.865	0.933 $\pm$ 0.287	5.136 $\pm$ 0.765
Thickness (μm)	10.825 $\pm$ 0.324	2.602 $\pm$ 0.606	11.098 $\pm$ 0.572	0.637 $\pm$ 0.118	8.656 $\pm$ 0.603	1.598 $\pm$ 0.269	8.888 $\pm$ 0.573

These effects were most dramatic in the putative lysozyme (Δr) and spannin ($\Delta r_z/r_z^1$) mutants, whose biofilms were reduced from wild-type levels by almost sevenfold with respect to biomass and 10-fold with respect to mean thickness. The biofilm of the putative holin mutant (Δs) was less attenuated and only manifested a threefold reduction in biomass (Fig. 1, Table 2) and a fivefold reduction in mean thickness (Fig. 1, Table 2). The mutants were also attenuated with respect to their ability to attach to PVC (Fig. 2a), and aggregated less than the wild-type (Fig. 2b). While the holin mutant (Δs) was compromised in its ability to autoaggregate, it was not as deficient as the putative lysozyme (Δr) and spannin

mutants ($\Delta r_z/r_z^1$). Complementation restored all phenotypes to near wild-type levels, except for the aggregation phenotype of Δs , which was only partially restored.

Transmission electron microscopy

To visualize the differences between mutants and wild-type PHL628, transmission electron microscopy images were obtained (Fig. 3). Electron micrographs of the wild-type *E. coli* PHL628 revealed a robust layer of curli on and around individual cells (Fig. 3a). In contrast, electron micrographs of the mutants revealed little or no curli around the cells (Fig. 3b–d).

Fig. 2. (a) Deletion of the DLP12 lysis genes (S , R , R_z/R_z^1) impairs cellular attachment to PVC when compared with wild-type PHL628 (WT). Overnight cultures were diluted and assayed for PVC attachment by CV staining (A_{595}) normalized to cell density (OD_{600}). The absolute values of A_{595} and OD_{600} for each strain are given above each bar. (b) Deletion of the DLP12 lysis genes (S , R , R_z/R_z^1) negatively affected autoaggregation when compared with wild-type PHL628 (WT). OD_{600} was measured from the top 5 mm of each culture and then again after the sample had been vortexed; values are reported as OD_{600} of the unvortexed samples compared with their vortexed counterparts. Error bars, SD.

Fig. 3. Transmission electron microscopy reveals that DLP12 mutants produce less curli than wild-type PHL628. Image captures of 300-mesh Formvar grids stained with 3% AMB for (a) wild-type PHL628, (b) ΔS mutant, (c) ΔR mutant and (d) $\Delta R_z/R_z^1$ mutant; arrows indicate stained curli fibres. (e) DLP12 lysis gene mutants (S , R , R_z/R_z^1) bind less Congo red (which binds curli) than wild-type PHL628 (WT). Congo red binding was quantified by A_{500} measurements normalized to culture density (OD_{600}). Error bars, SD.

Congo red assay

In order to obtain a more quantitative assessment of the apparent curli deficiency, mutant strains were subjected to a Congo red pull-down assay (Gophna *et al.*, 2001). Congo red binds in proportion to the amount of curli on the surface (Gophna *et al.*, 2001). A PHL628: $\Delta csgA$ mutant, deficient in the gene that encodes the curlin subunit, was constructed as a control. Although the lysis gene mutants were not as compromised for Congo red binding as the $\Delta csgA$ mutant, the assay revealed that all the mutants had significantly less curli ($P \leq 0.05$) than the wild-type (Fig. 3e).

Curli operon expression

To determine whether the lack of curli was caused by a reduction in transcription of the *csgBAC* operon, expression from the *csgB* promoter (P_{csgB}) was monitored using a short-lived P_{csgB} -GFP reporter (Junker, 2004). Expression was measured after 24 h of growth on MSM CA, since curli expression is highest in stationary phase cells (Barnhart & Chapman, 2006). Each of the mutants showed a marked decrease in GFP fluorescence normalized to cell density when compared with the wild-type. The ΔS mutant exhibited a fivefold decrease in P_{csgB} -derived fluorescence, whereas the ΔR and $\Delta R_z/R_z^1$ mutants showed a more dramatic effect, with a 17-fold and more than 20-fold decrease, respectively (Fig. 4). These differences were consistent with our microscopic observations, and suggested that the decrease in curli could best be explained by downregulation of curli expression in the mutants.

Cell wall and peptidoglycan assessments

Given the putative function of the lysis gene products, and the link between cell envelope status and curli gene expression (Barnhart & Chapman, 2006), we hypothesized that loss of the lysis genes might be affecting peptidoglycan status and thereby indirectly affecting curli production. Consistent with this hypothesis, the lysis genes did appear to affect cell wall status, as evidenced by the response of the mutants to SDS, a detergent that has frequently been used to interrogate cell wall status (Ize *et al.*, 2003; Walburger *et al.*, 2002). Fig. 5 shows that although the effect of growth in the presence of SDS was modest, the mutants lysed more than the wild-type control strain. Complementation with wild-type alleles restored the mutants to wild-type levels (Fig. 5).

We also found that peptidoglycan metabolism was altered in the three DLP12 mutants (Fig. 6). When we quantified the release of labelled peptidoglycan fragments from the cell walls of strains that had been grown in the presence of ^{14}C -labelled NAG, we found that neither the ΔS nor the ΔR mutant lost any ^{14}C label from its peptidoglycan over 15 h, whereas the wild-type lost more than 60% of its labelled NAG in the same time frame (Fig. 6), losing approximately 5% of the ^{14}C label per generation (data not shown). These results are consistent with other reports of peptidoglycan turnover in *E. coli* (Goodell & Schwarz, 1985; Hölting, 1998). Although the $\Delta R_z/R_z^1$ mutant did lose some labelled peptidoglycan, it still lost significantly less than the wild-type ($P < 0.05$). Once again, complementation restored the mutants to near wild-type levels. Since the mutants appeared to grow normally and showed no obvious morphological manifestations consistent with loss of peptidoglycan turnover, we reasoned that the mutants might simply be recycling their peptidoglycan more efficiently than the wild-type.

One anticipated consequence of increased peptidoglycan recycling was an increase in the intracellular concentration of *N*-acetylglucosamine 6-phosphate (NAG-6P). Earlier work by Barnhart & Chapman (2006) has shown that

Fig. 4. Expression of the genes encoding curli (*csgB*) is downregulated in the DLP12 lysis gene mutants (S, R, Rz/Rz¹) when compared with wild-type PHL628 (WT). Fluorescence for cells bearing the reporter vector pJBA110 carrying the *csgB* promoter (P_{csgB}) fused to a short-lived GFP was detected with a 485/20 excitation filter and a 530/25 emission filter and normalized to culture density (OD₆₀₀). Error bars, SD.

accumulation of NAG-6P results in decreased transcription of *csgBAC*, suggesting that NAG-6P acts as a signal molecule, regulating curli production via an unknown mechanism (Barnhart *et al.*, 2006). In order to address the possible connection between increased peptidoglycan recycling, NAG-6P and decreased curli expression, we deleted *nagK* in each of the lysis mutants. This gene encodes the only *E. coli* N-acetyl-D-glucosamine kinase and is required for the synthesis of NAG-6P (Uehara & Park, 2004). After deleting *nagK* in each of the DLP12 mutants we repeated the P_{csgB} -GFP reporter assay. Each of the DLP12 *nagK* lysis gene double-deletion mutants exhibited a dramatic and statistically significant ($P<0.01$) increase in curli transcription from the curli promoter (Fig. 7). There were 36-, 45- and 44-fold increases in curli transcription from the Δ*S* Δ*nagK*, Δ*R* Δ*nagK* and Δ*Rz/Rz*¹ Δ*nagK* strains, relative to the single deletion mutants, respectively. Interestingly, *nagK* deletion in the wild-type strain did not increase curli expression, suggesting that it is likely involved downstream of the impact of the DLP12 lysis cassette genes in regulating curli production (Fig. 7).

DISCUSSION

The results presented in this study provide new information on the importance of DLP12 lysis genes in biofilm formation by curli-producing *E. coli*. These mutants had altered biofilm architecture, biomass and mean thickness. Their biofilm attributes, coupled with the reduced attachment and autoaggregation phenotypes, are consistent with the observed decrease in curli expression, since curli are known to be important for cell-cell and cell surface interactions. (Gophna *et al.*, 2001; Vidal *et al.*, 1998). The electron micrographs and Congo red results confirmed reduced curli production by the mutants and suggest that the DLP12 lysis gene products somehow affect the display of amyloid fibres known to be involved in biofilm formation by some *E. coli* (Barnhart & Chapman, 2006).

These observations contrast markedly with those recently reported by Wang and co-workers, who found that the DLP12 lysis genes repressed biofilm production in *E. coli* BW25113, which is dependent on fimbriae for biofilm formation (Wang *et al.*, 2009). A more recent report

Fig. 5. Deletion of the DLP12 lysis genes (S, R, Rz/Rz¹) increases sensitivity to SDS challenge when compared with wild-type PHL628 (WT). Survival is reported as the ratio of the OD₆₀₀ of the cultures at *t*=3 h (OD_{600-3 h}/OD_{3 h}) divided by the starting OD₆₀₀ (OD₆₀₀₋₀/OD₀). Exogenous complementation of each of the genes shows SDS tolerance restored to wild-type levels. Absolute values for initial and 3 h OD₆₀₀ for each strain are given above the bars. Error bars, SD.

Fig. 6. Deletion of the DLP12 lysis genes (S, R, R_z/R_z^1) reduces the amount of peptidoglycan degradation products lost to the supernatant when compared with wild-type PHL628 (WT). Cells were grown in the presence of ^{14}C -labelled NAG for 12 h, then washed and diluted in fresh medium ($t=0$) with unlabelled NAG and processed at intervals over 15 h. Levels of ^{14}C label lost from SDS-boiled cells (a) after 15 h for all strains and their complements, or (b) over the course of 15 h for the wild-type PHL628 ($\diamond$) and ΔR mutant ($\blacksquare$). Error bars, SD.

Fig. 7. Deletion of *nagK* in the DLP12 lysis gene mutants increases curli expression to above wild-type PHL628 (WT) levels. Fluorescence for cells bearing the reporter vector pJBA110 carrying the *csgB* promoter (P_{csgB}) fused to a short-lived GFP was detected with a 485/20 excitation filter and a 530/25 emission filter, and normalized to culture density (OD_{600}). Error bars, SD.

(Wang *et al.*, 2010), however, has demonstrated that loss of the entire DLP12 prophage appears to reduce biofilm formation by BW25113; the reason for this discrepancy is not clear, but it may indicate that other DLP12 genes play an important role in this strain. With respect to the results presented here, we suggest that the contrasting results are due to the different mechanisms that these strains use to accomplish biofilm formation, since fimbriae expression is low in PHL628 and mannose had no effect on its aggregation properties (data not shown). Rather, we have provided evidence that the deletion of these lysis genes specifically results in the downregulation of curli in PHL628.

Although the exact reason for the downregulation of curli remains unclear, the results from our cell wall assessments with SDS and labelled NAG suggest that it may be an indirect response to cell wall stress caused by loss of any of the DLP12 lysis genes. Transcription of *csgBAC* is positively regulated by CsgD, which is part of the *csgDEFG* operon and is divergently transcribed from the intergenic region between it and the *csgBAC* locus. The regulation of *csgDEFG* transcription is controlled by at least eight different regulators (reviewed by Barnhart *et al.*, 2006), three of which (EnvZ/OmpR, CpxRA and RcsABCD) are known to respond to a wide range of envelope stresses (Barnhart & Chapman, 2006; Barnhart *et al.*, 2006).

Cell wall stress has been shown to result from the loss of active peptidoglycan-modifying enzymes (Ize *et al.*, 2003). Interestingly, however, no lysozyme activity has ever been reported for *E. coli* (Vollmer & Høltje, 2001); instead it uses lytic transglycosylases to degrade peptidoglycan during growth. The peptidoglycan degradation product of lytic transglycosylases (anhydro-*N*-acetylmuramic acid-*N*-acetylglucosamine) is known to be recycled, and yet peptidoglycan is still lost at a significant rate (5–10 % per generation) (Høltje, 1998; Keep *et al.*, 2006; Vollmer & Høltje, 2001). The exact nature of the carbon backbone of the unrecycled peptidoglycan degradation products, however, remains unknown (Goodell & Schwarz, 1985). It is possible that loss of the DLP12 lysozyme halts the production of nonrecyclable peptidoglycan degradation products: lysozymes produce only *N*-acetylmuramic acid-*N*-acetylglucosamine dimers, which are not recycled by *E. coli*. Our results, which are consistent with complete recycling of peptidoglycan degradation products by the DLP12 lysozyme mutant, suggest that the DLP12 lysozyme plays a role in liberating non-recyclable peptidoglycan degradation products from the sacculus.

Although we cannot yet point to a direct connection between altered peptidoglycan recycling and curli production, we have shown a link between production of NAG-6P and altered curli expression in the lysis mutants. It appears that NagK is a necessary factor for modulating the response of the cells to loss of the DLP12 lysis cassette with respect to *csgBAC* transcription. Intermediates in peptidoglycan synthesis and turnover have been shown to impact the

transcription of the genes encoding surface appendages such as fimbriae and curli (Barnhart & Chapman, 2006; Barnhart *et al.*, 2006; Vollmer & Tomasz, 2002; Vollmer & Høltje, 2004). Specifically, NAG-6P has been shown to inhibit expression of curli, although the exact mechanism remains unclear (Barnhart *et al.*, 2006). As both a building block and a breakdown product of peptidoglycan, NAG-6P would seem to be a useful signal molecule that might inform the cell of peptidoglycan status. Indeed, our data from the *nagK* lysis gene double mutants suggest that NAG-6P levels play a role in alerting the cell to the apparent stress caused by loss of the DLP12 lysis genes.

Clearly, more work is required to understand the full role of these phage remnants in *E. coli*; however, this report demonstrates that the DLP12 lysis genes are important for curli production and therefore biofilm formation, and suggests that they are not just genetic vestiges without a cellular function.

ACKNOWLEDGEMENTS

Dr Philip LeJeune kindly provided *E. coli* PHL628. This work was supported in part by grants from the American Heart Foundation and the United States Department of Agriculture.

REFERENCES

- Andersen, J. B., Sternberg, C., Poulsen, L. K., Bjorn, S. P., Givskov, M. & Molin, S. (1998). New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl Environ Microbiol* **64**, 2240–2246.
- Barnhart, M. M. & Chapman, M. R. (2006). Curli biogenesis and function. *Annu Rev Microbiol* **60**, 131–147.
- Barnhart, M. M., Lynem, J. & Chapman, M. R. (2006). GlcNAc-6P levels modulate the expression of curli fibers by *Escherichia coli*. *J Bacteriol* **188**, 5212–5219.
- Barrios, A. F., Zuo, R., Ren, D. & Wood, T. K. (2006). Hha, YbaJ, and OmpA regulate *Escherichia coli* K12 biofilm formation and conjugation plasmids abolish motility. *Biotechnol Bioeng* **93**, 188–200.
- Bayles, K. W. (2007). The biological role of death and lysis in biofilm development. *Nat Rev Microbiol* **5**, 721–726.
- Campbell, A. (1994). Comparative molecular biology of lambdoid phages. *Annu Rev Microbiol* **48**, 193–222.
- Casjens, S. (2003). Prophages and bacterial genomics: what have we learned so far? *Mol Microbiol* **49**, 277–300.
- Choi, K.-H. & Schweizer, H. P. (2005). An improved method for rapid generation of unmarked *Pseudomonas aeruginosa* deletion mutants. *BMC Microbiol* **5**, 30.
- Datsenko, K. A. & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640–6645.
- Dubrac, S., Boneca, I. G., Poupel, O. & Msadek, T. (2007). New insights into the WalK/WalR (YycG/YycF) essential signal transduction pathway reveal a major role in controlling cell wall metabolism and biofilm formation in *Staphylococcus aureus*. *J Bacteriol* **189**, 8257–8269.
- García-Contreras, R., Zhang, X.-S., Kim, Y. & Wood, T. K. (2008). Protein translation and cell death: the role of rare tRNAs in biofilm

formation and in activating dormant phage killer genes. *PLoS ONE* 3, e2394.

Genevaux, P., Muller, S. & Bauda, P. (1996). A rapid screening procedure to identify mini-Tn10 insertion mutants of *Escherichia coli* K-12 with altered adhesion properties. *FEMS Microbiol Lett* 142, 27–30.

Goodell, E. W. & Schwarz, U. (1985). Release of cell wall peptides into culture medium by exponentially growing *Escherichia coli*. *J Bacteriol* 162, 391–397.

Gophna, U., Barlev, M., Seijffers, R., Oelschlager, T. A., Hacker, J. & Ron, E. Z. (2001). Curli fibers mediate internalization of *Escherichia coli* by eukaryotic cells. *Infect Immun* 69, 2659–2665.

Heydorn, A., Nielsen, A. T., Hentzer, M., Sternberg, C., Givskov, M., Ersbøll, B. K. & Molin, S. (2000). Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* 146, 2395–2407.

Höltje, J.-V. (1998). Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. *Microbiol Mol Biol Rev* 62, 181–203.

Ize, B., Stanley, N. R., Buchanan, G. & Palmer, T. (2003). Role of the *Escherichia coli* Tat pathway in outer membrane integrity. *Mol Microbiol* 48, 1183–1193.

Jubelin, G., Vianney, A., Beloin, C., Ghigo, J. M., Lazzaroni, J. C., Lejeune, P. & Dorel, C. (2005). CpxR/OmpR interplay regulates curli gene expression in response to osmolarity in *Escherichia coli*. *J Bacteriol* 187, 2038–2049.

Junker, L. (2004). *Examination of biofilm development using array based and molecular approaches*. PhD dissertation, Cornell University, Ithaca, NY, USA.

Keep, N. H., Ward, J. M., Cohen-Gonsaud, M. & Henderson, B. (2006). Wake up! Peptidoglycan lysis and bacterial non-growth states. *Trends Microbiol* 14, 271–276.

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., II & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166, 175–176.

Lindsey, D. F., Mullin, D. A. & Walker, J. R. (1989). Characterization of the cryptic lambdoid prophage DLP12 of *Escherichia coli* and overlap of the DLP12 integrase gene with the tRNA gene *argU*. *J Bacteriol* 171, 6197–6205.

McCullar, M. V., Brenner, V., Adams, R. H. & Focht, D. D. (1994). Construction of a novel polychlorinated biphenyl-degrading bacterium: utilization of 3,4'-dichlorobiphenyl by *Pseudomonas acidovorans* M3GY. *Appl Environ Microbiol* 60, 3833–3839.

Pratt, L. A. & Kolter, R. (1999). Genetic analyses of bacterial biofilm formation. *Curr Opin Microbiol* 2, 598–603.

Prigent-Combaret, C., Prensier, G., Le Thi, T. T., Vidal, O., Lejeune, P. & Dorel, C. (2000). Developmental pathway for biofilm formation in curli-producing *Escherichia coli* strains: role of flagella, curli and colanic acid. *Environ Microbiol* 2, 450–464.

Rhodijs, V. A., Suh, W. C., Nonaka, G., West, J. & Gross, C. A. (2006). Conserved and variable functions of the σ^E stress response in related genomes. *PLoS Biol* 4, e2.

Ryu, J. H., Kim, H., Frank, J. F. & Beuchat, L. R. (2004). Attachment and biofilm formation on stainless steel by *Escherichia coli* O157:H7 as affected by curli production. *Lett Appl Microbiol* 39, 359–362.

Srividhya, K. V. & Krishnaswamy, S. (2007). Subclassification and targeted characterization of prophage-encoded two-component cell lysis cassette. *J Biosci* 32 (Suppl. 1), 979–990.

Stanley, N. R. & Lazazzera, B. A. (2004). Environmental signals and regulatory pathways that influence biofilm formation. *Mol Microbiol* 52, 917–924.

Uehara, T. & Park, J. T. (2004). The *N*-acetyl-D-glucosamine kinase of *Escherichia coli* and its role in murein recycling. *J Bacteriol* 186, 7273–7279.

Vidal, O., Longin, R., Prigent-Combaret, C., Dorel, C., Hooreman, M. & Lejeune, P. (1998). Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert surfaces: involvement of a new *ompR* allele that increases curli expression. *J Bacteriol* 180, 2442–2449.

Vollmer, W. & Höltje, J.-V. (2001). Morphogenesis of *Escherichia coli*. *Curr Opin Microbiol* 4, 625–633.

Vollmer, W. & Höltje, J.-V. (2004). The architecture of the murein (peptidoglycan) in Gram-negative bacteria: vertical scaffold or horizontal layer(s)? *J Bacteriol* 186, 5978–5987.

Vollmer, W. & Tomasz, A. (2002). Peptidoglycan *N*-acetylglucosamine deacetylase, a putative virulence factor in *Streptococcus pneumoniae*. *Infect Immun* 70, 7176–7178.

Walburger, A., Lazdunski, C. & Corda, Y. (2002). The Tol/Pal system function requires an interaction between the C-terminal domain of TolA and the N-terminal domain of TolB. *Mol Microbiol* 44, 695–708.

Wang, X., Kim, Y. & Wood, T. K. (2009). Control and benefits of CP4-57 prophage excision in *Escherichia coli* biofilms. *ISME J* 3, 1164–1179.

Wang, X., Kim, Y., Ma, Q., Hong, S. H., Pokusaeva, K., Sturino, J. M. & Wood, T. K. (2010). Cryptic prophages help bacteria cope with adverse environments. *Nat Commun* 1, 147.

Webb, J. S., Thompson, L. S., James, S., Charlton, T., Tolker-Nielsen, T., Koch, B., Givskov, M. & Kjelleberg, S. (2003). Cell death in *Pseudomonas aeruginosa* biofilm development. *J Bacteriol* 185, 4585–4592.

Webb, J. S., Lau, M. & Kjelleberg, S. (2004). Bacteriophage and phenotypic variation in *Pseudomonas aeruginosa* biofilm development. *J Bacteriol* 186, 8066–8073.

Whiteley, M., Banger, M. G., Bumgarner, R. E., Parsek, M. R., Teitzel, G. M., Lory, S. & Greenberg, E. P. (2001). Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* 413, 860–864.

Xu, M., Arulandu, A., Struck, D. K., Swanson, S., Sacchettini, J. C. & Young, R. (2005). Disulfide isomerization after membrane release of its SAR domain activates P1 lysozyme. *Science* 307, 113–117.

Young, I., Wang, I. & Roof, W. D. (2000). Phages will out: strategies of host cell lysis. *Trends Microbiol* 8, 120–128.

Zhang, N. & Young, R. (1999). Complementation and characterization of the nested Rz and Rz1 reading frames in the genome of bacteriophage lambda. *Mol Gen Genet* 262, 659–667.

Edited by: V. Sperandio