Sulfate assimilation pathway intermediate phosphoadenosine 5′-phosphosulfate acts as a signal molecule affecting production of curli fibres in *Escherichia coli*

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The enterobacterium *Escherichia coli* can utilize a variety of molecules as sulfur sources, including cysteine, sulfate, thiosulfate and organosulfonates. An intermediate of the sulfate assimilation pathway, adenosine 5′-phosphosulfate (APS), also acts as a signal molecule regulating the utilization of different sulfur sources. In this work, we show that inactivation of the *cysH* gene, leading to accumulation of phosphoadenosine 5′-phosphosulfate (PAPS), also an intermediate of the sulfate assimilation pathway, results in increased surface adhesion and cell aggregation by activating the expression of the curli-encoding *csgBAC* operon. In contrast, curli production was unaffected by the inactivation of any other gene belonging to the sulfate assimilation pathway. Overexpression of the *cysH* gene downregulated *csgBAC* transcription, further suggesting a link between intracellular PAPS levels and curli gene expression. In addition to curli components, the Flu, OmpX and Slp proteins were also found in increased amounts in the outer membrane compartment of the *cysH* mutant; deletion of the corresponding genes suggested that these proteins also contribute to surface adhesion and cell surface properties in this strain. Our results indicate that, similar to APS, PAPS also acts as a signal molecule, albeit with a distinct mechanism and role: whilst APS regulates organosulfonate utilization, PAPS would couple availability of sulfur sources to remodulation of the cell surface, as part of a more global effect on cell physiology.

INTRODUCTION

In natural environments, bacteria are exposed to sudden and drastic changes that can trigger specific responses (e.g. induction by a given nutrient of the genes involved in its utilization), but also induce more general effects on cell physiology responses, often involving regulation of motility and/or of cell adhesion factors (Mikkelsen *et al.*, 2011). For instance, availability of N-acetylglucosamine induces expression of the *nagBACD* operon and of the *nagE* gene, responsible for its uptake and degradation (Plumbridge, 1991), but it also modulates production of type 1 pili (Sohanpal *et al.*, 2004) and of curli fibres (Barnhart *et al.*, 2006).

In the repertoire of *Escherichia coli* adhesion factors, curli fibres (also known as thin aggregative fimbriae in *Salmonella*) represent a strong determinant for cell aggregation and surface adhesion (Prigent-Combaret *et al.*, 2000). Regulation of curli-encoding genes is extraordinarily complex and responds to a combination of environmental cues, such as low growth temperature (≤30°C), low osmolarity, slow growth and oxygen availability (Römling *et al.*, 1998a; Gerstel & Römling, 2001, 2003; Tagliabue *et al.*, 2010a). The signal molecules cAMP (Zheng *et al.*, 2004) and c-di-GMP (Kader *et al.*, 2006; Weber *et al.*, 2006), as well as intracellular concentration of pyrimidine nucleotides (Garavaglia *et al.*, 2012), also have a strong impact on curli gene expression. These environmental and physiological signals are relayed by a number of regulatory proteins, including RpoS, OmpR, IHF, H-NS, CpxR, Crl, CRP, MlrA and the MqsR/MqsA toxin/antitoxin system, which affect curli gene transcription either directly or indirectly (Bougour *et al.*, 2004; Brown *et al.*, 2001; Gerstel *et al.*, 2004; Hung *et al.*, 2001; Prigent-Combaret *et al.*, 2001; Römling *et al.*, 1998a; Soo & Wood, 2013). In addition, at least five non-coding RNAs have been

Abbreviations: APS, adenosine 5′-phosphosulfate; EPS, extracellular polysaccharide; MudPIT, multidimensional protein identification technology; PAP, adenosine 3′,5′-bisphosphate; PAPS, phosphoadenosine 5′-phosphosulfate; q, quantitative; RT, real-time.

One supplementary table and two supplementary figures are available with the online version of this paper.
shown to take part in post-transcriptional regulation of the curli-related operon csgDEFG (Mika & Hengge, 2013). Thus, curli fibre production appears to be a major target for environmental signals promoting cell adhesion.

Sulfur is an essential element and is typically provided in bacterial growth media at millimolar concentrations (Neidhardt et al., 1974). In addition to being a component of the amino acids cysteine and methionine, sulfur is present in reducing compounds (e.g. hydrogen sulfide and glutathione) that play an important role in maintaining the redox conditions of the bacterial cytoplasm and in detoxification of reactive oxygen species (Toledano et al., 2007). E. coli can utilize a wide range of sulfur sources, both inorganic (e.g. sulfate, sulfite, thiosulfate) and organic (e.g. cysteine, methionine, organosulfonates), with cysteine being the preferred substrate (van der Ploeg et al., 2001). However, due to its abundance in nature, sulfate is thought to be the main source of sulfur for E. coli in most environments. Once taken up by the bacterium, sulfate is readily reduced to hydrogen sulfide by the sulfate assimilation pathway, whose first steps involve activation of sulfate through the formation of two modified nucleotides: adenosine 5′-phosphosulfate (APS) and phosphoadenosine 5′-phosphosulfate (PAPS) (Liu et al., 1994) (Fig. 1). Preferential order in sulfur source utilization is achieved through the activity of two regulatory proteins: CysB, which activates the sulfate assimilation pathway in the absence of exogenous cysteine (Kredich, 1992), and Cbl, whose activation of the organosulfonate utilization genes is counteracted by APS (Bykowski et al., 2002). Such a finely tuned regulatory circuitry determines a clear hierarchy in utilization of sulfur sources: cysteine>sulfate>organosulfonates.

In this study, we provide evidence that inactivation of the cysH gene, encoding PAPS reductase, induces overproduction of curli fibres and of other outer membrane proteins, reshaping cell-surface-associated structures. We propose that the effects of the cysH mutation are mediated by intracellular PAPS accumulation; thus, PAPS would act as a signal molecule allowing E. coli to sense changes in the availability of different sulfur sources and to adapt its physiology accordingly.

**METHODS**

**Bacterial strains and growth conditions.** Bacterial strains used in this work are listed in Table 1. For strain construction and manipulation, bacteria were grown in LB medium (10 g tryptone 1 L⁻¹, 5 g yeast extract 1 L⁻¹, 5 g NaCl 1 L⁻¹). If required, antibiotics were added at the following concentrations: ampicillin, 100 μg ml⁻¹; chloramphenicol, 50 μg ml⁻¹; kanamycin, 50 μg ml⁻¹; tetracycline, 25 μg ml⁻¹.

For Congo red- and calcofluor-binding assays, overnight cultures were spotted, using a replicator, on either Congo red- or calcofluor-supplemented agar media. Both media were modifications of the yeast extract-Casamino acid (YESCA) medium (Pratt & Silhavy, 1996), and were composed of 1% Casamino acids, 0.15% yeast extract, 0.005% MgSO₄, 0.0005% MnCl₂, 2% agar to which either 0.004% Congo red and 0.002% Coomassie blue (for Congo red medium) or 0.005% calcofluor (for calcofluor medium) were added after autoclaving. Bacteria were grown for either 24 h at 30 °C or 18 h at 37 °C; phenotypes were better detectable after a further 24–48 h incubation at 4 °C.

For gene expression regulation studies, bacteria were grown in YESCA liquid medium composed of 1% Casamino acids, 0.15% yeast extract, 0.005% MgSO₄, 0.0005% MnCl₂. Surface adhesion to polystyrene microtitre plates was analysed on bacterial cultures grown overnight in YESCA at 30 and 37 °C, and adhesion units were determined as described previously (Dorel et al., 1999).

To verify cysteine auxotrophy, bacteria were grown for 24–48 h at 37 °C in either M9 (Carzaniga et al., 2012) or sulfate-free M9 (SF-M9) media with or without supplementation with 0.25 mM cysteine. SF-M9 was obtained substituting MgSO₄ with MgCl₂ at a final concentration of 1 mM. Glucose (0.4%) was added as sole carbon source to obtain M9/Glu or SF-M9/Glu media. Growth media were inoculated (1:500) with bacteria from overnight cultures in LB medium, after centrifugation and resuspension in PBS to OD₆₀₀ 1.0.

**Plasmid construction.** Plasmids and primers used in this work are listed in Tables 1 and S1 (available in the online Supplementary Material), respectively. For expression of WT CysH protein, the corresponding gene was amplified by PCR from the E. coli MG1655 chromosome using primers cysH_NdeI_for and cysH_PstI_rev, and the resulting product was cloned into pT7-7 vector using the Ndel/PstI restriction sites. The pT7-cysHmut plasmid, carrying a mutant cysH allele encoding a protein with a non-functional redox-active centre (Berendt et al., 1995), was constructed by amplifying the cysH gene using primers cysH_Ndel_for and cysH_mut_PstI_rev, resulting in the following substitutions: T→G at nt 715, G→C at nt 716, C→G at nt 724, A→C at nt 725. The four mutations resulted in substitution of both Cys240 and His243 to alanine residues (ECGLH→EAGLA). The obtained PCR product was cloned into the pT7-7 vector as the WT allele using the Ndel/PstI restriction sites. Both the WT and mutant alleles of the cysH gene were verified by sequencing.

**Mutant construction.** Transposon insertion mutagenesis was carried out using the EZ-Tn5 <R6K<ori/KAN-2> Transposome kit (Epicentre). Transposon mutagenesis and determination of transposon insertion site by rescue cloning were carried out according to the manufacturer’s instructions. E. coli MG1655 mutant derivatives were constructed using the Δ Red technique (Datsenko & Wanner, 2000). Primers used for gene inactivation and for confirmation of target gene disruption by PCR are listed in Table S1.

**Gene expression assays.** Gene expression levels were measured through quantitative real-time (qRT)-PCR as described previously (Gualdi et al., 2007) using 16S RNA as reference gene. RNA was extracted from overnight cultures grown in YESCA medium at 30 or 37 °C in full aeration, achieved by constant shaking at 100 r.p.m. The complete list of primers used for amplification is reported in Table S1.

**Analysis of the outer membrane compartment.** For proteomic analysis, outer membrane-protein-enriched samples were obtained using the N-lauroylsarcosinate method as described previously (Gualdi et al., 2007). Enriched samples were treated with RapiGest SF (Waters) and digested with trypsin as described previously (Comunian et al., 2011). Digested samples were then analysed by 2D micro-liquid chromatography coupled to ion-trap MS (2DC-MS/MS) using the ProteomeX-2 configuration (Thermo Electron), as described previously (Palma et al., 2010). The experimental mass spectra produced

http://mic.sgmjournals.org
Fig. 1. Sulfate reduction and cysteine biosynthesis pathways in *E. coli* (adapted from http://ecocyc.org). The modified nucleotide PAPS accumulating in the MG1655ΔcysH mutant is highlighted in green. The cysH gene is highlighted in red. Underlined genes belong to the CysB regulon (Kredich, 1992). More details are provided in the text. PAP, adenosine 3',5'-bisphosphate.
Table 1. *Escherichia coli* strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>MG1655</td>
<td>K-12, F− λ− ilvG− rfb-50 rph-1</td>
<td>Blattner et al. (1997)</td>
</tr>
<tr>
<td>EB1.3</td>
<td>MG1655 rpoS::tet</td>
<td>Prigent-Combaret et al. (2001)</td>
</tr>
<tr>
<td>AM70</td>
<td>MG1655 ΔcsgA::cat</td>
<td>Tagliabue et al. (2010b)</td>
</tr>
<tr>
<td>AM75</td>
<td>MG1655ΔcsgD::cat</td>
<td>Tagliabue et al. (2010a)</td>
</tr>
<tr>
<td>MG1655ΔcysH::Tn5Kan</td>
<td>Tn5::kan transposon inserted at nt 472 of the cysH gene coding sequence</td>
<td>This work</td>
</tr>
<tr>
<td>MG1655ΔcysH</td>
<td>Replacement of the cysH gene with a chloramphenicol resistance cassette</td>
<td>This work</td>
</tr>
<tr>
<td>MG1655ΔcysB</td>
<td>Replacement of the cysB gene with a chloramphenicol resistance cassette</td>
<td>This work</td>
</tr>
<tr>
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<td>Replacement of the cysU gene with a chloramphenicol resistance cassette</td>
<td>This work</td>
</tr>
<tr>
<td>MG1655ΔcysD</td>
<td>Replacement of the cysD gene with a chloramphenicol resistance cassette</td>
<td>This work</td>
</tr>
<tr>
<td>MG1655ΔcysC</td>
<td>Replacement of the cysC gene with a chloramphenicol resistance cassette</td>
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<td>This work</td>
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<tr>
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<td>Replacement of the ompX gene with a kanamycin resistance cassette</td>
<td>This work</td>
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<tr>
<td>MG1655Δslp</td>
<td>Replacement of the slp gene with a kanamycin resistance cassette</td>
<td>This work</td>
</tr>
<tr>
<td>MG1655Δflu</td>
<td>Replacement of the ompX gene with a tetracycline resistance cassette</td>
<td>This work</td>
</tr>
<tr>
<td>MG1655ΔcysHΔcsgA</td>
<td>Obtained by bacteriophage P1 transduction from AM70 to MG1655ΔcysH::Tn5Kan</td>
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<tr>
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<tr>
<td>MG1655ΔcysHΔcsgD</td>
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</tr>
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<td>This work</td>
</tr>
<tr>
<td>MG1655ΔcysHΔslp</td>
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<td>This work</td>
</tr>
<tr>
<td>MG1655ΔcysHΔflu</td>
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<td>This work</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT7-7</td>
<td>Ampicillin resistance, T7 RNA polymerase-dependent promoter</td>
<td>Gualdi et al. (2007)</td>
</tr>
<tr>
<td>pT7cysHmut</td>
<td>cysH gene cloned into pT7-7 vector as a 735 bp NdeI/PstI fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pT7cysHmut</td>
<td>cysH allele carrying the mutation resulting in the ECGLH→EAGLA change in the CysH redox site</td>
<td>This work</td>
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</tbody>
</table>

by MudPIT (multidimensional protein identification technology) analyses were correlated with tryptic peptide sequences by comparison with theoretical mass spectra obtained by in silico digestion of the *E. coli* K-12 MG1655 protein database. Outputs were treated with an in-house software called MAProMa (multidimensional algorithm protein map) (Mauri & Deho, 2008) to identify differentially expressed proteins. Different protein expression was estimated by means of the DAve (differential average) algorithm of MAProMa (Mauri et al., 2005). A DAve value of either >0.1 or <−0.1 is an indication of different relative expression levels between two samples, whilst a DAve value of either 200 or −200 indicates the exclusive presence of a protein in one sample.

Extracellular polysaccharide (EPS) determination was carried out by total sugar quantification using the phenol/H2SO4 method as described previously (Dubois et al., 1956).

**Statistical analysis.** If not otherwise stated, all experiments were performed at least in triplicate and the data were analysed by one-way ANOVA, with a P value of 0.05 being significant, using the statistical software package R (R Development Core Team, 2013).

**RESULTS**

Inactivation of the cysH gene affects the production of extracellular structures

Congo red and calcofluor dyes provide an easy way to identify the production of both proteinaceous and EPS surface-exposed structures. Congo red binds with high affinity to amyloid fibres, such as curli fibres (Hammar et al., 1995), and with lower affinity to cellulose (Teather & Wood, 1982) and poly-N-acetylglucosamine (Romeo, 1998; Carzaniga et al., 2012); calcofluor binds to various EPS components, in particular cellulose and chitin, resulting in fluorescence detectable by UV light (Harrington & Raper, 1968). In order to identify novel genes involved in the production and regulation of extracellular structures, we performed transposon mutagenesis in the *E. coli* K-12 strain MG1655: mutants were then screened for their phenotype on Congo red and calcofluor medium at both 30 °C, a...
temperature permissive for curli production, and 37 °C, a
temperature at which production of curli fibres does not
take place under standard growth conditions (Römling et al.,
1998a). Out of several mutants showing altered phenotypes
on both media, one mutant displaying a dark-red phenotype
at 30 °C, a red coloration at 37 °C on Congo red and
increased fluorescence on calcofluor, was further charac-
terized. Mapping of the EZ-Tn5 <R6Kcoryl/KAN-2> transposon indicated that the insertion site lay at nt 472
of the 735 nt cysH gene, encoding PAPS reductase. The
location of the transposon insertion site suggested inac-
tivation of the cysH gene. To verify this hypothesis, we
constructed a cysH mutant in which the complete coding
sequence of the gene was deleted (MG1655ΔcysH). As
expected, the MG1655ΔcysH mutant displayed the same
phenotypes as the MG1655cysH::Tn5-kan strain, i.e. red
and fluorescent on Congo red and calcofluor media,
respectively (Fig. 2a), and was therefore used in further
experiments.

PAPS reductase catalyses the conversion of PAPS into
adenosine 3',5'-bisphosphate (PAP) and sulfite, in the
sulfate reduction pathway leading to sulfur assimilation
and cysteine biosynthesis (Fig. 1). Indeed, both transposon
insertion and cysH deletion led, as expected, to cysteine
auxotrophy, as shown by the inability of either cysH
mutant to grow in M9/Glu minimal medium with sulfate
as the sole sulfur source (Table 2). Addition of
0.25 mM cysteine to M9/Glu fully overcame the growth
defect (Table 2). The effects of cysH inactivation on cell-
surface-associated structures could thus be ascribed to
cysteine depletion. Indeed, starvation for various nutrients
represents an important cue for the induction of the
biosynthesis of extracellular structures involved in cellular
aggregation and biofilm formation (Gerstel & Römling,
2001). However, MG1655ΔcysH showed the same growth
rate as its parental strain in liquid YESCA (Fig. S1), which,
like the Congo red and calcofluor solid media, contains
cysteine from the added Casamino acids and yeast extract,
suggesting that the cysH mutant was not subjected to
cysteine starvation under the conditions used for our
phenotypic assays.

To further investigate whether the phenotypic effects due
to the cysH inactivation could be dependent on either
cysteine starvation or accumulation of PAPS, or of any
other intermediate in the sulfate assimilation pathway,
we constructed deletion mutants in the cysU, cysD, cysC
and cysI genes, involved in each step of sulfate reduction
to hydrogen sulfide (Fig. 1), and in the cysB gene, which
encodes a positive regulator of the genes belonging to
the sulfate assimilation and cysteine biosynthesis pathways
(Kredich, 1992). As expected, all mutants were auxotrophic
for cysteine (Table 2); however, they displayed WT
phenotypes on Congo red and calcofluor plates, with the
partial exception of the cysQ mutant, showing some
fluorescence on calcofluor medium (Fig. 2a). The beha-
viour of the mutants in the sulfate assimilation pathway
confirmed that the phenotypes observed in cysH mutant
strains were not due to cysteine starvation, but depended
on the loss of a functional CysH protein. It could thus be
hypothesized that lack of PAPS reductase activity by CysH
would cause intracellular PAPS accumulation that, in turn,
might result in the Congo red and calcofluor phenotypes
of the MG1655ΔcysH strain. To further confirm this
hypothesis, we complemented a cysH mutant with plasmids

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**Fig. 2.** (a) Congo red (CR) and calcofluor (CF) binding by E. coli strains deficient in the sulfate reduction pathway. MG1655 and isogenic mutants in the cysB, cysU, cysD, cysC, cysH and cysI genes were spotted on both media and grown at either 30
(for 24 h) or 37 °C (for 18 h). Plates were incubated at 4 °C for at least 48 h to enhance dye binding. (b) Complementation of
MG1655ΔcysH with plasmids carrying WT (pT7cysHwt) and mutant (pT7cysHmut) alleles of the cysH gene. pT7-7, vector.
Table 2. Growth of mutants in genes belonging to the sulfate assimilation pathway in minimal medium with or without cysteine supplementation.

Growth was measured as OD₆₀₀ on stationary-phase cultures grown for 24 h at 37 °C with vigorous shaking (>100 r.p.m.) in either M9/Glu or sulfate-free M9/Glu (SF-M9/Glu) media in the presence or absence of 0.25 mM cysteine. Values are the means of three separate experiments with very similar results.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth (OD₆₀₀)</th>
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<tbody>
<tr>
<td></td>
<td>M9/Glu</td>
</tr>
<tr>
<td></td>
<td>No additions</td>
</tr>
<tr>
<td>MG1655</td>
<td>1.43</td>
</tr>
<tr>
<td>MG1655cysH::Tn5-kan</td>
<td>0.01</td>
</tr>
<tr>
<td>MG1655ΔcysB</td>
<td>0.02</td>
</tr>
<tr>
<td>MG1655ΔcysU</td>
<td>0.02</td>
</tr>
<tr>
<td>MG1655ΔcysD</td>
<td>0.02</td>
</tr>
<tr>
<td>MG1655ΔcysC</td>
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<tr>
<td>MG1655ΔcysH</td>
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<tr>
<td>MG1655ΔcysI</td>
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<td>MG1655ΔcysH/pT7cysHwat</td>
<td>1.62</td>
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<tr>
<td>MG1655ΔcysH/pT7cysHmut</td>
<td>0.02</td>
</tr>
</tbody>
</table>

NT, not tested.

carrying either a WT cysH gene or a mutant allele, in which two codons encoding C-terminal amino acids identified as the redox-active centre of the enzyme (Berendt et al., 1995) had been substituted to obtain two alanine residues (pT7cysHwt and pT7cysHmut, respectively). Production of both the WT and mutated CysH proteins from the pT7-7 plasmid derivatives was clearly detectable on SDS-PAGE (Fig. S2); indeed, although the pT7-7 vector relied on T7 bacteriophage RNA polymerase for gene overexpression, background expression levels were fairly high, probably due to the presence of promoter-like sequences recognized by E. coli RNA polymerase (Brombacher et al., 2006). As expected, the mutated CysH protein was not able to rescue MG1655ΔcysH auxotrophy for gene overexpression, background expression levels were fairly high, probably due to the presence of promoter-like sequences recognized by E. coli RNA polymerase (Brombacher et al., 2006). As expected, the mutated CysH protein was not able to rescue MG1655ΔcysH auxotrophy for gene overexpression, background expression levels were fairly high, probably due to the presence of promoter-like sequences recognized by E. coli RNA polymerase (Brombacher et al., 2006). As expected, the mutated CysH protein was not able to rescue MG1655ΔcysH auxotrophy for gene overexpression, background expression levels were fairly high, probably due to the presence of promoter-like sequences recognized by E. coli RNA polymerase (Brombacher et al., 2006).

Effects of the cysH mutation on cell-surface-associated structures.

The red phenotype on Congo red medium shown by the MG1655ΔcysH mutant was suggestive of increased curli production, whilst calcofluor fluorescence, although usually linked with EPS synthesis, could also depend on curli overproduction, via direct calcofluor binding either to curli fibres or to some curli-associated factor (Uhlich et al., 2014). Indeed, determination of cell-surface-associated polysaccharides did not show any significant differences in EPS amounts between MG1655 and its cysH derivative (data not shown), thus suggesting that, in this strain, calcofluor fluorescence was not dependent on EPS. As curli production strongly enhances cell aggregation and adhesion to solid surfaces (Vidal et al., 1998; Cookson et al., 2002), we tested the MG1655ΔcysH mutant for its ability to adhere to an abiotic surface. Microtitre adhesion assay revealed a moderate (around twofold), but highly reproducible increase in biofilm formation by the MG1655ΔcysH strain at 30 °C (Fig. 3b), whilst no significant differences between strains were observed at 37 °C (data not shown). Phase-contrast microscopy observations provided further confirmation that cysH inactivation stimulated the production of aggregative structures, which, again, were more common in cultures grown at 30 than at 37 °C (Fig. 3c).

As curli fibres are not produced in E. coli K-12 strains at 37 °C, these results were consistent with stimulation of curli production by inactivation of the cysH gene. Indeed, an MG1655ΔcysHΔcsgA double mutant, unable to produce curli, was impaired in both surface adhesion and cell aggregation (Fig. 3b; data not shown). However, the MG1655ΔcysHΔcsgA mutant still displayed a red phenotype on Congo red and fluorescence on calcofluor medium at both 30 and at 37 °C (Fig. 3a), and a slight increase in surface adhesion when compared with an MG1655ΔcsgA strain (Fig. 3b). This result suggested that cysH inactivation...
promoted the production of an additional factor, able to bind Congo red and calcofluor at both temperatures, and to act as a weak cell aggregation factor.

In order to identify extracellular factors other than curli whose production could be influenced by cySH inactivation, we performed protein expression analysis by using MudPIT on an outer membrane-protein-enriched fraction. As the ΔcySH-dependent phenotype was observed at both 30 and 37 °C, we focused on proteins showing differential regulation at both temperatures; only a relatively small protein set fulfilled these criteria (Table 3). Protein analysis experiments showed clearly that curli production was increased as a result of cySH inactivation; indeed, the main curli subunit CsgA, as well as the CsgF and CsgG proteins, involved in curli assembly and secretion, were present at higher levels in the MG1655ΔcySH strain. Although enhanced production of curli proteins occurred at both 30 and 37 °C, Csg proteins were detected at lower levels at 37 than at 30 °C even in MG1655ΔcySH (Table 3), in agreement with microscopy observations and surface adhesion experiments (Fig. 3c and data not shown), suggesting that the cySH mutation could not fully override temperature-dependence of curli expression.

In addition to curli-related proteins, three more proteins were produced at higher levels in MG1655ΔcySH: Slp, a lipoprotein induced under carbon starvation or during the stationary phase (Alexander & St John, 1994), OmpX, an outer membrane protein with unknown function, but related to virulence and to EPS production (Mecsas et al., 1995; Vogt & Schulz, 1999; Otto & Hermansson, 2004), and Flu, also known as Antigen 43, which can mediate cell–cell aggregation and reduce cell motility (Danese et al., 2000; Ulett et al., 2006). To investigate whether any of the identified proteins could be responsible for the MG1655ΔcySH phenotypes, we inactivated the corresponding genes in both MG1655 and MG1655ΔcySH. Although none of the three mutations resulted in a straightforward reversion to the WT phenotypes, they all resulted in a partial loss of either Congo red or calcofluor binding (or both) in the cySH mutant background (Fig. 4a). Inactivation of either the slp or flu genes also impaired cell adhesion to polystyrene, albeit exclusively in the...
### Table 3. List of upregulated proteins at 30 and 37 °C in the outer membrane-protein-enriched fraction of MG1655ΔcysH

<table>
<thead>
<tr>
<th>GenBank accession no.</th>
<th>Protein name</th>
<th>Notes</th>
<th>Score*</th>
<th>DAve</th>
<th>30 °C</th>
<th>37 °C</th>
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<td>MG1655 MG1655</td>
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<td></td>
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<td>MG1655 MG1655</td>
<td>Curli-encoding proteins</td>
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<td>NP_415560 CsgA</td>
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*Score and DAve are both algorithms of the MAProMa software (Mauri & Deho, 2008). Score is a function of the number of uniquely identified peptides in each sample. DAve provides a relative expression ratio between the two samples. Values of 0.1 or –0.1 indicate differential relative expression level between two samples; a DAve value of either 200 or –200 indicates the exclusive presence of a protein in one sample.

MG1655ΔcysH background (Fig. 4b). Thus, our results strongly suggest that, in addition to being produced at higher levels in MG1655ΔcysH, the OmpX, Flu and Slp proteins contribute to its cell surface properties, such as Congo red and calcofluor binding and surface adhesion.

### Inactivation of the cysH gene activates transcription of the csgBAC operon

We investigated whether increased curli production observed in the MG1655ΔcysH strain could be mediated by gene expression regulation of curli-encoding genes. The csgDEFG operon, which codes for proteins that take part in curli assembly and secretion, and for the transcription regulator CsgD, is the hub of a complex regulatory network (Römling et al., 2000; Gerstel & Römling, 2003; Gerstel et al., 2004; Mika & Hengge, 2013). The CsgD protein, in turn, activates the csgBAC operon, encoding curli subunits. Using qRT-PCR, we determined the transcript levels of csgD and csgB, representative of the two curli-encoding operons. cysH inactivation resulted in only a slight increase in csgD expression (~1.7-fold induction; data not shown). In contrast, csgB transcription was strongly stimulated in the MG1655ΔcysH mutant at both 30 and 37 °C (29- and 23-fold, respectively; Fig. 5a), in agreement with higher levels of CsgA protein (encoded by the second gene of the csgBAC operon) in the outer membrane fraction at both temperatures. csgBAC upregulation in the cysH mutant strain did not bypass the requirement for CsgD; indeed, csgB activation was almost completely lost in a MG1655ΔcysHAΔcsgD double mutant (Fig. 5a). In contrast to csgB, expression levels for flu, ompX and slp genes were not altered in MG1655ΔcysH (data not shown), suggesting that the higher amounts of their gene products in the outer membrane preparations of this strain (Table 3) did not depend on transcription regulation of their corresponding genes.

To further confirm the role of the CysH protein in curli gene expression, we measured transcript levels of the csgB and csgD genes in the MG1655 strain overexpressing the cysH gene from the pT7cysH<sub>wt</sub> plasmid (Fig. 5b). Consistent with the results observed in the MG1655ΔcysH mutant, cysH overexpression caused a significant downregulation (fivefold) of the csgB gene, whilst only having a slight effect on csgD transcript levels (1.5-fold, Fig. 5b).

As qRT-PCR experiments strongly suggested that upregulation of csgB transcription in MG1655ΔcysH still required CsgD (Fig. 5a), we investigated whether the Congo red and calcofluor phenotype of the MG1655ΔcysH mutant could also be dependent on this regulator. In addition, we considered a possible role for the alternative sigma factor σ<sup>32</sup>, which, in addition to being necessary for csgD transcription (Römling et al., 1998b), also controls expression of the slp gene (Shimada et al., 2004) and of several genes responsible for biosynthesis of c-di-GMP (Sommerfeldt et al., 2009) – a signal molecule playing a pivotal role in regulation of cell-surface-associated structures (Tamayo et al., 2007). Finally, we tested the possibility that Cbl, the regulator of
the organosulfonate utilization pathway, whose activity is mediated by APS, could also be the receptor protein for PAPS. In order to investigate the possible roles of CsgD, RpoS and Cbl, we inactivated the genes encoding for these regulators in the MG1655ΔcysH background (Fig. 5c); consistent with their involvement in the regulation of factors responsible for the MG1655ΔcysH phenotypes, inactivation of either csgD or rpoS affected to different extents, but did not completely abolish, Congo red and calcofluor binding. In contrast, inactivation of the cbl gene did not affect the Congo red and calcofluor phenotypes in either the MG1655ΔcysH strain (Fig. 5c) or in MG1655 (data not shown), suggesting that the Cbl protein did not mediate PAPS-dependent signalling.

**DISCUSSION**

The ability to sense and utilize sulfur sources appears to be tightly connected to surface adhesion and biofilm formation in *E. coli*. Indeed, mutations affecting *cysB*, encoding the master regulator of the sulfate assimilation pathway, and the *cysE* gene, whose product synthesizes O-acetylserine, i.e. the inducer of CysB activity, have been shown to trigger biofilm formation (Sturgill et al., 2004; Ren et al., 2005). Likewise, inactivation of genes involved in sulfate assimilation stimulated biofilm formation in rich medium (glucose-supplemented LB; Domka et al., 2007). In addition, CysB has also been reported to control production of the siderophore pyoverdin – an important virulence factor in *Pseudomonas aeruginosa* (Imperi et al., 2010). However, mutations affecting the *cysB* regulatory gene, or the sulfate assimilation pathway as a whole, likely result in general perturbation of cell physiology; indeed, *cysB* can impact carbon fluxes and modulate cAMP production (Quan et al., 2002) – processes known to strongly impact biofilm formation in *E. coli* (Romeo et al., 1993; Jackson et al., 2002).

In this work, we have shown that inactivation of the *cysH* gene, but of no other genes in the sulfate assimilation pathway, results in enhanced cell aggregation and surface adhesion in *E. coli*. Deletion of the *cysH* gene affects outer-membrane protein composition, promoting the production of Slp, OmpX and Flu proteins, and of curli fibres (Figs 2–4, Table 3). The increase in curli fibre production involves transcription activation of the *csgBAC* operon (Fig. 5). We propose that *cysH* inactivation alters outer-membrane protein patterns and cell-surface-associated structures due to accumulation of PAPS – the substrate of the CysH (PAPS reductase) protein (Fig. 1). This modified nucleotide could act as a signal molecule by relaying to the bacterial cell, through the activity of the sulfate assimilation pathway, the availability of different sulfur sources. In this respect, PAPS would complement APS, the other modified nucleotide present as intermediates in the sulfate assimilation pathway (Fig. 1); APS binds the regulatory protein Cbl, preventing transcription activation of genes involved in uptake and metabolism of organosulfonates, and thus allowing preferential utilization of sulfate as a sulfur source (Bykowski et al., 2002). Thus, both modified nucleotides found as intermediates in the

![Figure 4](https://example.com/fig4.jpg)

**Fig. 4.** (a) Effects of *slp*, *ompX* and *flu* gene inactivation in the MG1655ΔcysH strain on Congo red (CR) and calcofluor (CF) binding at 30 and 37 °C. Strains were grown at either 30 (for 24 h) or at 37 °C (for 18 h). Plates were incubated for 48 h at 4 °C to enhance Congo red and calcofluor binding. (b) Surface adhesion of *slp*, *ompX* and *flu* mutants to polystyrene microtitre plates. The adhesion unit values, assessed as described previously (Dorel et al., 1999), are the mean ± se of at least four independent experiments.
sulfate assimilation pathway would act as signal molecules, although targeting different processes; whilst APS controls the utilization of sulfur sources, PAPS might have a broader activity, such as the remodulation of outer membrane proteins and extracellular structures in response to sulfur source availability.

Genetic evidence would suggest that neither CysB nor Cbl, i.e. the two regulatory proteins directly related to sulfur assimilation, are the direct target for PAPS (Figs 2a and 5c, and data not shown); thus, the molecular mechanisms of PAPS-dependent regulation remain to be identified. However, it can be speculated that PAPS accumulation in the MG1655ΔcysH mutant might favour translation efficiency of the csgDEFG mRNA, which would be consistent with the higher amounts of the CsgF and CsgG detected in the outer membrane fraction of MG1655ΔcysH (Table 3), and with activation of csgB transcription (Fig. 5a) due to increased production of its regulator, the CsgD protein. Several modified nucleotides are involved in post-transcriptional regulation, often as cofactors of enzymes involved in RNA processing and turnover, e.g. c-di-GMP and ppGpp modulate RNase activity by polynucleotide phosphorylase in *E. coli* and in actinomycetes, respectively (Siculella et al., 2010; Tuckerman et al., 2011). Interestingly, *E. coli* oligoribonuclease (Orn), involved in degradation of short RNA fragments, is inhibited by a PAP, i.e. the product of CysH PAPS reductase activity (Mechold et al., 2006). However, depletion of PAP does not seem to be responsible for the phenotypes observed in MG1655ΔcysH, as mutants blocked at the upstream steps of the sulfate assimilation

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**Fig. 5.** (a) Relative expression levels of the csgB gene in strains MG1655, MG1655ΔcysH, MG1655ΔcsgD and MG1655ΔcysHΔcsgD measured through qRT-PCR on overnight cultures (–18 h) grown at 30 (grey bars) and 37 °C (black bars). The csgB expression value in MG1655 was set to 1. Data are the mean ± se of at least three independent experiments. (b) Effects of cysH overexpression on csgB and csgD transcripts in the MG1655 WT strain. The MG1655 strain was transformed with the pT7-7 vector, either empty (pT7-7) or carrying the WT allele of the cysH gene (pT7cysHwt). The csgB and csgD expression values in MG1655 transformed with pT7-7 were set to 1. The reported values are the mean ± sd of at least two experiments performed on three RNA samples extracted from independent cultures. (c) Effects of inactivation of regulatory genes csgD, rpoS and cbl on Congo red (CR) and calcofluor (CF) binding. Strains were grown at either 30 (for 24 h) or 37 °C (for 18 h). Plates were incubated for 48 h at 4 °C to enhance Congo red and calcofluor binding.
pathway also do not produce PAP. In addition, the cysQ mutant, unable to convert PAP to AMP and phosphate, shows a weak fluorescent phenotype on calciofluor medium (Fig. 2a), probably due to inhibition of PAPS reductase activity of the CysH protein by PAP (Berendt et al., 1995), leading to PAPS accumulation. A regulatory role for PAPS in cellular processes not related to sulfur metabolism is also suggested by the observations that PAPS (and also PAP) can inhibit the enzyme nucleotide diphosphate kinase (Ndk) (Schneider et al., 1998), thus affecting intracellular nucleotide pools, and that CgrA, a protein carrying a PAPS-binding domain, participates in transcription regulation of cup fimbrial genes in P. aeruginosa (McManus & Dove, 2011). Future experiments will be aimed at the characterization of direct and indirect effects of PAPS accumulation, at the identification of additional targets, and at the understanding of the molecular mechanisms involved in this process.

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REFERENCES


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