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 (\( \leq 30°C \)), 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 (Bougdour 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 Cbi, 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 l\(^{-1}\), 5 g yeast extract l\(^{-1}\), 5 g NaCl 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, 1998), and were composed of 1% Casamino acids, 0.15% yeast extract, 0.005% MgSO\(_4\), 0.0005% MnCl\(_2\), 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% MgSO\(_4\), 0.0005% MnCl\(_2\) 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 MgSO\(_4\) with MgCl\(_2\) 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 OD\(_{600}\) 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-cysH\(_{mut}\) 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\(_{NdeI}\)_for and cysH\(_{PstI}\)_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 \(<\text{R6K}^\text{ori}/\text{KAN-2}>\) 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 \(\lambda\) 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-lauroyl sarcosinate 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...
**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>
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</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1655</td>
<td>K-12, F−, \lambda−, ilvG−, rfb-50, rph-1</td>
<td>Blattner et al. (1997)</td>
</tr>
<tr>
<td>EB1.3</td>
<td>MG1655 rpoS::tct</td>
<td>Prigent-Combaret et al. (2001)</td>
</tr>
<tr>
<td>AM70</td>
<td>MG1655ΔcsgA::cat</td>
<td>Tagliahue et al. (2010b)</td>
</tr>
<tr>
<td>AM75</td>
<td>MG1655ΔcsgD::cat</td>
<td>Tagliahue 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>
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<tr>
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<td>Replacement of the cysU gene with a chloramphenicol resistance cassette</td>
<td>This work</td>
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<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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<tr>
<td>MG1655ΔcysI</td>
<td>Replacement of the cysI gene with a chloramphenicol resistance cassette</td>
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<tr>
<td>MG1655ΔcysQ</td>
<td>Replacement of the cysQ gene with a kanamycin resistance cassette</td>
<td>This work</td>
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<tr>
<td>MG1655ΔompX</td>
<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>
<td>This work</td>
</tr>
<tr>
<td>MG1655ΔcysHΔpooS</td>
<td>Obtained by bacteriophage P1 transduction from MG1655ΔcysH to EB1.3</td>
<td>This work</td>
</tr>
<tr>
<td>MG1655ΔcysHΔcsgD</td>
<td>Obtained by bacteriophage P1 transduction from MG1655ΔcysH::Tn5Kan to AM75</td>
<td>This work</td>
</tr>
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<td>MG1655ΔcysHΔcbl</td>
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<tr>
<td>MG1655ΔcysHΔompX</td>
<td>Obtained by replacing the ompX gene with a kanamycin resistance cassette in MG1655ΔcysH</td>
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<tr>
<td>MG1655ΔcysHΔslp</td>
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<td>This work</td>
</tr>
<tr>
<td>MG1655ΔcysHΔflu</td>
<td>Obtained by replacing the flu gene with a tetracycline resistance cassette in MG1655ΔcysH</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></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>pT7cysH&lt;sub&gt;mut&lt;/sub&gt;</td>
<td>cysH gene cloned into pT7-7 vector as a 735 bp Ndel/PstI fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pT7cysH&lt;sub&gt;mut&lt;/sub&gt;</td>
<td>cysH allele carrying the mutation resulting in the ECGLH→EAGLA change in the CysH redox site</td>
<td>This work</td>
</tr>
</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/H<sub>2</sub>SO<sub>4</sub> 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 characterized. Mapping of the EZ-Tn5 <R6KURY1/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 inactivation 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). 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 behaviour 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

![Fig. 2.](image-url)
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 OD_{600} 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>M9/Glu</th>
<th>SF-M9/Glu</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No additions</td>
<td>Cys</td>
</tr>
<tr>
<td>MG1655</td>
<td>1.43</td>
<td>1.27</td>
</tr>
<tr>
<td>MG1655cysH::Tn5-kan</td>
<td>0.01</td>
<td>1.32</td>
</tr>
<tr>
<td>MG1655ΔcysB</td>
<td>0.02</td>
<td>1.17</td>
</tr>
<tr>
<td>MG1655ΔcysU</td>
<td>0.02</td>
<td>1.27</td>
</tr>
<tr>
<td>MG1655ΔcysD</td>
<td>0.02</td>
<td>1.20</td>
</tr>
<tr>
<td>MG1655ΔcysC</td>
<td>0.01</td>
<td>1.25</td>
</tr>
<tr>
<td>MG1655ΔcysH</td>
<td>0.02</td>
<td>1.33</td>
</tr>
<tr>
<td>MG1655ΔcysI</td>
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<td>1.53</td>
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<tr>
<td>MG1655ΔcysH/pT7-7</td>
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<td>NT</td>
</tr>
<tr>
<td>MG1655ΔcysH/pT7cysH_{mut}</td>
<td>1.62</td>
<td>NT</td>
</tr>
<tr>
<td>MG1655ΔcysH/pT7cysH_{mut}</td>
<td>0.02</td>
<td>NT</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 (pT7cysH_{mut} and pT7cysH_{mut}, 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 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 cysteine (Table 2), demonstrating its inability to carry out PAPS reductase activity. Likewise, only the WT cysH allele was able to restore WT phenotypes on Congo red and calcofluor medium (Fig. 2b), suggesting strongly that loss of PAPS reductase activity by the CysH protein, leading to intracellular PAPS accumulation, was indeed responsible for cell surface structure alteration in MG1655ΔcysH. We attempted to quantify intracellular PAPS concentrations in both the MG1655 strain and its cysH mutant derivative using HPLC and MS; unfortunately, neither technique was able to provide a precise PAPS estimation due to the difficulty in distinguishing PAPS from other adenylic nucleotides (data not shown).

**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

Fig. 3. (a) Phenotypes on Congo red (CR) and calcofluor (CF) media of MG1655 (WT), MG1655ΔcsgA, MG1655ΔcysH and MG1655ΔcysHΔcsgA strains. 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. (b) Surface adhesion of the same set of strains to polystyrene microtitre plates. The adhesion unit values, assessed as described previously (Dorel et al., 1999), are the mean±s.e of at least four independent experiments. Letters provide the representation for post hoc comparisons. According to post hoc analysis (Tukey’s honest significant difference, P<0.05), bars sharing the same letter are not significantly different from each other. (c) Phase-contrast micrographs (main picture, ×400 magnification; inset, ×1000 magnification) of MG1655 and MG1655ΔcysH strains grown 16 h in YESCA medium at either 30 or 37 °C. The images were acquired with a digital CCD Leica DFC camera.
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† (MG1655ΔcysH/MG1655)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MG1655</td>
<td>MG1655ΔcysH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30 °C</td>
<td>37 °C</td>
</tr>
<tr>
<td>Curli-encoding proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP_415560</td>
<td>CsgA</td>
<td>Major curlin subunit</td>
<td>85.34</td>
<td>10.29</td>
</tr>
<tr>
<td>NP_415555</td>
<td>CsgG</td>
<td>Curli production assembly/transport component</td>
<td>50.28</td>
<td>30.22</td>
</tr>
<tr>
<td>NP_415556</td>
<td>CsgF</td>
<td>Curli production assembly/transport component</td>
<td>65.24</td>
<td>10.2</td>
</tr>
<tr>
<td>Other outer membrane proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP_417963</td>
<td>Slp</td>
<td>Starvation lipoprotein</td>
<td>35.24</td>
<td>55.25</td>
</tr>
<tr>
<td>NP_415335</td>
<td>OmpX</td>
<td>Outer membrane protein X</td>
<td>70.26</td>
<td>80.26</td>
</tr>
<tr>
<td>YP_026164</td>
<td>Flu</td>
<td>Autotransporter Antigen 43</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

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.
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 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
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
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 calcofluor 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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