L-Cysteine is required for induced antibiotic resistance in actively swarming *Salmonella enterica* serovar Typhimurium

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Swarm-cell differentiation in *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) results in a biosynthetic mode of growth, despite growing on a rich medium, and cells that have elevated antibiotic resistance. These phenotypes are not a prerequisite for swarm motility. By blocking the switch to anabolic growth using amino acid auxotrophs and screening for the presence of elevated antibiotic resistance in the swarm state, we found that cysteine biosynthesis is crucial for complete swarm-cell differentiation. Mutants were made in each *cys* biosynthetic operon and all had decreased antibiotic resistance in the swarm state, while swim-cell resistance remained the same as that of wild-type cells. This swarm-state-specific decreased resistance in Δ *cys* strains could be restored to wild-type levels by the addition of cysteine to swarm medium. Two regulatory mutants, Δ *cysB* and Δ *cysE*, failed to swarm unless cysteine was supplemented to the medium. We show that all CysB-responsive operons involved in cysteine biosynthesis are upregulated in the swarm state, even though swarm cells are cultivated on a medium that represses cysteine biosynthesis in the swim state. While swarm medium has sufficient cysteine for growth of *S. typhimurium*, it does not contain enough for swarm-cell differentiation. We hypothesize that in these cells, the additional cysteine requirement is for use in pathways not directly related to cell growth.

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INTRODUCTION

Bacteria exhibit a wide variety of motility mechanisms and behaviours (Harshey, 2003). Free swimming behaviour is most commonly associated with flagellated bacteria; however, many of these bacteria are also capable of moving over a surface. Swarming is a form of coordinated surface motility performed by some flagellated bacteria when grown on semi-solid media. Vegetative cells undergo a differentiation programme to produce swarm cells, which are typically elongated, hyper-flagellated and multi-nucleate (Fraser & Hughes, 1999; Harshey, 2003). While motility is required for cells to swarm, chemotaxis is not essential (Burkart *et al.*, 1998). Swarming was first described, and has been most studied, in *Proteus mirabilis* (Harshey, 2003; Henrichsen, 1972). In this species, swarm cells align along their long axis and migrate in multi-cellular rafts on agar concentrations up to 2% (Fraser & Hughes, 1999). *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) can swarm on nutrient-rich media supplemented with a rich carbon source, and having an agar concentra-

tion ranging from 0.5% to 0.8% (Burkart *et al.*, 1998; Kim & Surette, 2003; Toguchi *et al.*, 2000).

In addition to the motility phenotype, swarming has been associated with important physiological changes in numerous bacterial species. Virulence factor expression, notably urease, protease and haemolysin production, is increased in *Proteus mirabilis* in the swarm state, as is the ability to invade urinary epithelial cells (Allison *et al.*, 1992a, b; Liaw *et al.*, 2003). In *S. typhimurium*, actively migrating swarm cells have increased antibiotic resistance (Kim *et al.*, 2003), a switch in central metabolism from catabolic to anabolic growth (Kim & Surette, 2004), and increased ability to sense acylhomoserine lactones (Kim & Surette, 2006). The increased resistance of *S. typhimurium* swarm cells has been observed to many classes of antibiotics with a broad range of cellular targets and is not due to a genetic change (Kim *et al.*, 2003). Swarm cells transferred to liquid media reacquire the antibiotic sensitivity of vegetative cells (Kim & Surette, 2004). Furthermore, since swarm cells are growing exponentially, increased resistance is not due to slow growth or the presence of persister cells (Balaban *et al.*, 2004; Kim & Surette, 2004). Rather, swarm populations are

Abbreviations: NAS, *N*-acetyl-L-serine; OAS, *O*-acetyl-L-serine; RLU, relative light unit(s).

an example of an induced intrinsic antibiotic resistance whereby a genetically identical population that is normally sensitive to the action of an antibiotic becomes transiently more resistant (Levin & Rozen, 2006). To be induced, this phenotype does not require the presence of antibiotics (Kim & Surette, 2004).

S. typhimurium can assimilate inorganic sulfur into sulfide for the incorporation of reduced sulfur into cysteine (Fig. 1) (Peck, 1961). The final reaction of cysteine biosynthesis is catalysed by one of the isozymes encoded by *cysK* or *cysM*. CysK is produced at high levels and is thought to be the primary enzyme responsible for this reaction (Kredich, 1996); however, CysM is the predominant enzyme catalysing the reaction in anaerobic conditions, and when thiosulfate is present to react with *O*-acetyl-L-serine (OAS) (Kredich, 1996). The enzyme encoded by *cysQ* is thought to degrade a toxic intermediate of this pathway (Neuwald *et al.*, 1992). Three separate transport systems have been identified for cysteine transport: CTS-1, CTS-2 and CTS-3 (Baptist & Kredich, 1977). CTS-1, a part of the CysB regulon, accounts for the majority of cysteine uptake, and is repressed by growth on cysteine and other reduced sulfur sources. CTS-2 and CTS-3 are lower-capacity transporters of cysteine than CTS-1, although CTS-2 has a higher affinity for cysteine (Baptist & Kredich, 1977). Sulfate and thiosulfate are imported by the same permease as encoded by the genes *cysU-cysW-cysA* but having different periplasmic binding proteins, *sbp* and *cysP*, respectively (Hryniewicz & Kredich, 1991).

Expression of genes involved in cysteine biosynthesis is controlled by the LysR-type transcriptional regulator CysB. CysB binds the inducer, *N*-acetyl-L-serine (NAS), the product of a non-enzymic rearrangement of OAS (Sturgill *et al.*, 2004). Serine transacetylase (CysE) is feedback inhibited by cysteine, providing kinetic control of the production of cysteine through OAS. Sulfide and thiosulfate are potent anti-inducers of the CysB regulon, by antagonizing binding of NAS to CysB (Hryniewicz & Kredich, 1991; Ostrowski & Kredich, 1990). Operons involved in the cysteine biosynthetic pathway in the *S. typhimurium* CysB regulon include *cysPUWAM*, *cysDNC*, *cysJIH*, *cysQ* and *cysK* (Fig. 1). CysB binds to NAS and activates transcription at each operon. For maximal expression of the CysB regulon, sulfur limitation is required to relieve end-product inhibition of CysE by cysteine and allow the synthesis of OAS (Kredich, 1996).

This study was initiated by asking whether the swarm phenotypes of metabolic differentiation and elevated antibiotic resistance were coupled. To block metabolic differentiation, amino acid auxotrophs were generated. These mutants were subsequently screened in the swarm state for the presence of increased antibiotic resistance phenotype. From this initial screen, it became apparent that cysteine biosynthesis was crucial for complete swarm-cell differentiation, as only cysteine auxotrophs did not have increased antibiotic resistance in the swarm state. This

Fig. 1. Sulfur assimilation and cysteine biosynthesis pathway in *S. typhimurium*. The shaded area indicates the operon organization and the CysB regulon. CysB positively regulates the genes in sulfur assimilation and cysteine biosynthesis when bound with NAS. Evidence presented in this study suggests that *cysE* may be activated by CysB. Abbreviations: APS, adenosine 5'-phosphosulfate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; OAS, *O*-acetyl-L-serine; NAS, *N*-acetyl-L-serine.

led us to further study cysteine biosynthesis in swarm cells. We investigated expression of operons encoding cysteine biosynthetic enzymes using transcriptional fusions. Mutants in each *cys* locus were generated to allow study of antibiotic resistance and swarm colony morphologies in cysteine auxotroph swarm colonies. We investigated the regulatory mechanism by which swarm cells were able to induce the *cys* pathway despite growing on medium containing sufficient reduced sulfur, which usually represses cysteine biosynthesis in swim cells. The mutant studies were confirmed using a chemical genetics approach to induce cysteine auxotrophy in wild-type cells. As swarm medium contains sufficient cysteine to support growth and swarming of cysteine auxotrophs, we hypothesize that the increased cysteine requirement is a prerequisite for complete swarm-cell differentiation in *S. typhimurium*.

METHODS

Plasmids, strains, media and growth conditions. Bacterial strains, and plasmids used in this study are listed in Table 1. For routine culture, strains were grown in Lennox's Luria-Bertani broth (LB) at 37 °C, with shaking (200 r.p.m.). Swarm medium NBG

Table 1. Bacterial strains, and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
Bacterial strains		
14028	Wild-type <i>S. typhimurium</i>	ATCC
<i>cysD</i>	14028 <i>cysD</i> ::Tn10dCm	This study
<i>cysN</i>	14028 <i>cysN</i> ::Tn10dCm	This study
<i>cysH</i>	14028 <i>cysH</i> ::Tn10dCm	This study
<i>cysP</i>	14028 <i>cysP</i> ::Tn10dCm	This study
<i>cysQ</i>	14028 <i>cysQ</i> ::Tn10dCm	This study
<i>cysIJ</i>	14028 Δ <i>cysIJ</i> ::Cm	This study
<i>cysE</i>	14028 Δ <i>cysE</i> ::Cm	This study
<i>cysK</i>	14028 Δ <i>cysK</i> ::Cm	This study
<i>cysB</i>	14028 Δ <i>cysB</i> ::Cm	This study
<i>cysC</i>	14028 Δ <i>cysC</i> ::Cm	This study
<i>cysK cysM</i>	14028 Δ <i>cysK</i> :: Δ Cm Δ <i>cysM</i> :: Δ Cm	This study
Plasmids		
pSig70-16	pCS26 containing σ^{70} -responsive promoter	K. Pabbaraju, University of Calgary
pCysD	pCS26 containing <i>cysD</i> operon promoter	This study
pCysJ	pCS26 containing <i>cysJ</i> operon promoter	This study
pCysQ	pCS26 containing <i>cysQ</i> promoter	This study
pCysG	pCS26 containing <i>cysG</i> promoter	This study
pCysP	pCS26 containing <i>cysP</i> operon promoter	This study
pCysB	pCS26 containing <i>cysB</i> promoter	This study
pCysK	pCS26 containing <i>cysK</i> promoter	This study

(Nutrient Broth, Difco, with 0.5 % glucose, 0.5 % agar) and swim medium NBG (Nutrient Broth with 0.5 % glucose, 0.25 % agar) were used in swarm and swim assays, respectively, as previously described (Kim & Surette, 2003). M9 minimal medium was from Difco. Kanamycin was used at 50 $\mu\text{g ml}^{-1}$, and chloramphenicol was used at 12.5 $\mu\text{g ml}^{-1}$. Sulfate, sulfite, sulfide, thiosulfate, L-cysteine and 1,2,4-triazole (Sigma-Aldrich) were added as indicated.

Transposon mutants were generated using a P22 random transduction of a Tn10dCm library of *S. typhimurium*, as previously described (Maloy, 1990). The site of the chloramphenicol resistance gene, *cat*, insertion was determined using arbitrary-primed PCR. Mutants obtained from this technique were *cysD*::Tn, *cysN*::Tn, *cysH*::Tn, *cysQ*::Tn and *cysP*::Tn.

Mutant strains Δ *cysC*, Δ *cysIJ*, Δ *cysE*, Δ *cysK*, Δ *cysK cysM* and Δ *cysB* were constructed using the λ Red system (Datsenko & Wanner, 2000). Primers used for the generation of PCR products are listed in Table 2. The antibiotic resistance gene was resolved from both sites in the Δ *cysK cysM* double mutant as described by Datsenko & Wanner (2000).

Construction of transcriptional fusions. PCR primers used for the construction of transcriptional fusions are listed in Table 2. All promoters were cloned from *S. typhimurium* 14028 genomic DNA by PCR. Each pair of PCR primers for amplifying a promoter contained an *XhoI* and a *BamHI* site and amplified products were cloned into pCS26, a low-copy vector containing a promoterless luciferase operon (*luxCDABE*), as described previously (Bjarnason *et al.*, 2003). All enzymes used in cloning were purchased from Invitrogen and molecular biology techniques were conducted using standard techniques (Sambrook & Russell, 2001). Cloned promoters were verified by DNA sequencing.

Luciferase assays. Cells were harvested from the periphery of swarm or swim colonies using sterile toothpicks and transferred to

400 μl volumes of PBS. One hundred microlitres of cells in PBS were measured in triplicate, with a 1 s read time, using a Trilux Scintillation Counter (Wallac). Each strain was repeated in triplicate from a separate overnight culture, used to inoculate three swarm plates for each condition. Remaining cells in PBS were serially diluted and plated on Luria-Bertani (LB) agar with kanamycin to determine c.f.u. in each sample. Luciferase activity, in counts per second (c.p.s.), was normalized to 10^7 c.f.u. ml^{-1} . This value is referred to as relative light units (RLU), with 1 RLU equalling 1 c.p.s. per 10^7 c.f.u.

Luminescent images of swarm and swim plates were taken using an Alpha Imager FluorChem 8900 CCD camera (Alpha Innotech) with no exogenous light and using an exposure of 10 s to 5 min to capture light emitted by the luciferase reporter.

Antibiotic resistance measurements. Antibiotic resistance on swarm and swim media was determined by placing an E-Test strip (AB Biodisk) in the centre of the plate as described previously (Kim *et al.*, 2003; Kim & Surette, 2003). Briefly, 1 μl of overnight culture was spotted on either side of the strip adjacent to the strip. Plates were incubated at 37 °C and the antibiotic resistance was read according to the manufacturer's instructions after the swarm colony covered the plate. The conventional method of streaking cells onto a plate, resulting in a confluent layer of growth, is not suitable for measuring antibiotic resistance in swarm cells. Broth-grown cells spread plated on swarm medium cannot actively swarm, thus not allowing measurement of antibiotic resistance in this population of cells.

Composition analysis of Nutrient Broth (NB). HPLC analysis was performed by the Biochemical Genetics Laboratory at the Alberta Children's Hospital under the direction of Dr Floyd F. Snyder. Samples were reduced with 0.5 M DTT. Proteinase K digestion was performed on NB (40 g l^{-1}). After autoclaving, filter-sterilized proteinase K (Invitrogen) was added to a final concentration of 400 mg l^{-1} to one flask. To the control flask, the same volume of sterile water was added. These flasks were incubated with stirring for

Table 2. PCR primers used in the construction of promoter fusions and deletion mutants

Primer	Sequence
CysJR1	CATCCGGCAATTCAGGTTATTCACAGAAATCACGCGGGGTGTAGGCTGGAGCTGCTTC
CysJR2	GATCCGCTTTGCTTACTGGAACATAACGACGCATGACGACCCTCCTTAGTTCCTATTCCG
CysBR1	TGCCATGCCACTACGACACAAACCGACGGTGATATTACTTGTGTAGGCTGGAGCTGCTTC
CysBR2	GATGGCCTGATGGCGCTAATCTGGATGATGATTATGAAACCTCCTTAGTTCCTATTCCG
CysER1	TCGGAACAGCGTTTTTTAGTTGTACCGCGCAATTCAGATGGTGTAGGCTGGAGCTGCTTC
CysER2	AACGGGTTGGTCGTTTTCTGCCGCTCTGGAGTAAGCCATGCCTCCTTAGTTCCTATTCCG
CysKR1	AGGTATCCCAATTTTCATACAGTTAAGGACAGGCCATGAGTGTGTAGGCTGGAGCTGCTTC
CysKR2	AGGTGCTTTTTTACGCGTTTTTAACATGCTGGCATCACTGCCTCCTTAGTTCCTATTCCG
CysCR1	TCAGGATCTGATAATATCGCGCCGTCTCAGCAGGTCTAATGTGTAGGCTGGAGCTGCTTC
CysCR2	CATGGCGCTGCATGATGAGAACGTGGTCTGGCACTCTCATCTCCTTAGTTCCTATTCCG
CysM1	GCCTGTGTTTATCCCAATCAGCTGAACTCATTAGTTTAAAGTGTAGGCTGGAGCTGCTTC
CysM2	CTACGCATACCGGGCTTTTTTACGAGCTGAAAACGTGAATCCTCCTTAGTTCCTATTCCG
CysD1	AGTCCTCGAGCATAATGAGTCCTTAAATAC
CysD2	AGTCGGATCCTAACCGTTTCTTGAATAC
CysJ1	AGTCGGATCCGCGTCGTTATGTTCCAGTAAGCAAAG
CysJ2	AGTCCTCGAGCGTGGTGGACATGATGAACCTCTCAG
CysQ1	AGTCCTCGAGAAGCGTTGCACTAACTTAATC
CysQ2	AGTCGGATCCATTCTCCACCTCTTTATGAC
CysG1	AGTCCTCGAGCACGGTACTGTTCTTCAAAG
CysG2	AGTCGGATCCTATAGGCAAATGGTCCACGAC
CysP1	AGTCGGATCC TCTTTTTCAGTAAGTTAACGGC
CysP2	AGTCCTCGAGAGCGTAGGCGTCTGATTGAT
CysE1	AGTCCTCGAG TCGAAGGCTATCGCAATACG
CysE2	AGTCGGATCCCGCGGGCTTCAGCTTAAATATTC
CysB1	AGTCGGATCCCTGCTGCAATTCATAATAC
CysB2	AGTCCTCGAGTTGAAGGCAAGAAATAACCC
CysK1	AGTCCTCGAGAGCCTCTTTACGATGATTCC
CysK2	AGTCGGATCCATAGTCAGCGAGTTATCTTC

12 h at 37 °C, after which time both flasks were autoclaved again to inactivate the proteinase K. This sample was then analysed by HPLC.

RESULTS

Cysteine auxotrophs retain swarm motility and lose the induced antibiotic resistance phenotype

Actively swarming *Salmonella* differentiate into a unique metabolic state characterized by a biosynthetic mode of growth and elevated antibiotic resistance (Kim & Surette, 2004). If these two phenotypes are physiologically coupled, we reasoned that blocking biosynthetic growth may interfere with the antibiotic resistance phenotype. We hypothesized that auxotrophs would be forced to take up an amino acid from the medium and therefore would be incapable of undergoing full metabolic differentiation. Amino acid auxotrophs were generated using P22 transposon mutagenesis and were examined for motility and the elevated antibiotic resistance swarm phenotypes. In the initial screen, 23 auxotrophs in nine different biosynthetic pathways were examined (data not shown). All mutants displayed wild-type swim motility, chemotactic behaviour and swarm motility (data not shown). This is consistent with previous genetic screens that did not identify any

auxotrophs in screens for swarm motility mutants (Kim *et al.*, 2003). The auxotrophs were tested for resistance to gentamicin and ciprofloxacin under swim and swarm conditions. All auxotrophs displayed antibiotic resistance similar to wild-type swarm cells, with the exception of cysteine auxotrophs. Each cysteine auxotroph examined showed decreased antibiotic resistance in the swarm state. Although this screen was not exhaustive of all amino acids, it demonstrated the importance of cysteine in swarm-cell differentiation. An auxotroph for methionine, another sulfur-containing amino acid, behaved like the wild-type, showing the characteristic elevated resistance to antibiotics in the swarm state (data not shown).

In the vegetative (swim) state, the cysteine auxotrophs showed similar susceptibility to antibiotics as wild-type cells. However, under swarm growth conditions, while the cysteine auxotrophs retained swarm motility, the ability to induce elevated antibiotic resistance was lost.

Cysteine auxotrophs have decreased antibiotic resistance in the swarm state

To further explore the link between cysteine auxotrophs and induced antibiotic resistance, mutants were generated in all *cys* operons. To test the relationship between the

cysteine biosynthetic pathway and the induced antibiotic resistance of swarm cells, each auxotrophic strain was tested for susceptibility to gentamicin and ciprofloxacin using E-Test strips (Table 3). All mutants retained their ability to swarm with the exception of the regulatory mutants $\Delta cysE$ and $\Delta cysB$. For the *cys* mutants that retained swarm motility, each had increased susceptibility to at least one antibiotic tested. Ciprofloxacin MIC values for cysteine auxotrophs were 188- to 255-fold lower than wild-type (Table 3). The pattern of resistance to gentamicin was similar, with most cysteine auxotrophs demonstrating >3- to >8-fold decreased MIC compared to wild-type (Table 3). Obtaining absolute ratios between auxotrophs and wild-type was not possible for gentamicin because the value for the wild-type exceeded the maximum MIC value on the strip ($256 \mu\text{g ml}^{-1}$).

The susceptibility of *cysP* and *cysK* mutants to gentamicin was similar to that of wild-type swarm cells, which exceeded the maximum value on the E-test strip (Table 3). However, this *cysK* strain is not a cysteine auxotroph since CysK and CysM are functionally redundant, although this strain grows poorly on intermediates of the sulfate assimilation pathway, such as sulfide. In the absence of CysK, CysM can produce cysteine from sulfide or thiosulfate and OAS (Kredich, 1996). Having one functional O-acetylserine thiolase may allow an intermediate level of swarm-cell differentiation between the full differentiation observed in wild-type cells and the less differentiated cysteine auxotrophs. A *cysP* deletion mutant is also not an auxotroph; however, the *cysP* strain used in this study is polar on downstream genes in the operon, causing auxotrophy due to lack of a functional ABC transporter for sulfate and thiosulfate (Sirko *et al.*, 1995).

Cysteine auxotrophs have altered swarm colony morphology

Closer analysis of swarm colonies of the cysteine auxotroph mutants revealed altered morphologies that were distinct

from wild-type (Fig. 2). The mutant swarm morphologies fell into three categories. *cysP*, *cysH* and *cysI* mutants have a migrating swarm population that forms tendrils that dichotomously branch and terminate without intercepting, and also lack the complex circular structure surrounding the site of inoculation (Fig. 2b).

Mutants in *cysD*, *cysC* and *cysN*, genes that are all in one operon, had swarm colonies that resembled each other, but were distinct from wild-type (Fig. 2c). This group is typified by thick growth at the site of inoculation and complete lack of a circular structure surrounding the site of inoculation. This structure in wild-type cells consists of a mixture of swim and differentiating swarm cells, and the site of inoculation has only a thin layer of bacterial growth.

As noted above, two mutants in the regulatory genes, *cysE* and *cysB*, do not swarm using the standard swarm medium

Fig. 2. Swarm colony morphologies of *S. typhimurium* 14028 and *cys* mutants on NBG swarm medium (left column) and on NBG swarm medium supplemented with 2 mM cysteine (right column). Swarm plates were photographed after 9 h incubation at 37 °C. Swarm colony morphologies are shown for (a) 14028, (b) *cysI*::Tn, (c) *cysD*::Tn and (d) $\Delta cysB$.

Table 3. MICs ($\mu\text{g ml}^{-1}$) of gentamicin (GM) and ciprofloxacin (CI) for swarm cultures, determined by E-test strips

Strain	MIC of GM		MIC of CI	
	Swim	Swarm	Swim	Swarm
WT	12	>256	0.047	12
<i>cysP</i>	12	>256	0.064	0.016
<i>cysK</i>	24	>256	0.125	0.023
<i>cysN</i>	8	96	0.094	0.064
<i>cysD</i>	16	96	0.094	0.064
<i>cysH</i>	12	32	0.064	0.064
<i>cysC</i>	24	32	0.094	0.064
<i>cysI</i>	12	32	0.094	0.047
<i>cysQ</i>	16	48	0.094	0.064
10 mM triazole	12	96	0.094	0.064

(Fig. 2d). After 24 h, when wild-type cells have covered a Petri dish, these strains are the size of a swarm colony after approximately 5 h.

Swarm phenotypes of cysteine auxotrophs could be complemented by addition of 0.2 mM cysteine to the swarm medium, making the swarm colony morphologies of all cysteine auxotrophs indistinguishable from wild-type (Fig. 2). The addition of cysteine also allowed *cysB* and *cysE* mutants to swarm. Furthermore, the addition of 0.2 mM cysteine to the swarm medium restored antibiotic resistance of each auxotrophic strain to wild-type levels (data not shown), indicating that antibiotic resistance of cysteine auxotrophic swarm cells can be attributed to cysteine starvation.

The CysB regulon is induced in swarm cells, and these cells remain responsive to environmental inorganic sulfur source

To examine the different requirements for cysteine in swarm and swim cells, we measured transcription of genes in the CysB regulon in both cellular states using promoter–*luxCDABE* fusions. Gene expression was monitored by imaging *lux* expression on swim and swarm plates. This also produced spatial information on gene expression (Fig. 3a). With the exception of *cysK* and *sbp*, mutants in all other CysB-regulated genes showed little or no activity on swim plates. However, on swarm plates expression of all *cys* operons was induced.

To quantitatively measure gene expression, the level of *lux* expression was determined by measuring c.p.s. in a sample of swarm cells using a Trilux scintillation counter and normalizing expression values to cell number. This allowed comparison of gene expression between swarm and swim cells. A synthetic σ^{70} promoter (Kim & Surette, 2006) was used as a control in the gene expression experiments to demonstrate that overall transcriptional activity in the cell did not vary between the swarm and swim states. This reporter had a swarm-to-swim ratio of 0.9 (Fig. 3b). *cysB* was expressed at low constitutive levels in both the swim and swarm states; however, expression was consistently slightly higher in swarm cells. As *cysB* encodes a transcriptional regulator, a large increase in expression is not expected, since a small change in expression may have large consequences on transcription of its regulon. Consistent with results above, only *sbp* and *cysK* were expressed above background levels. *sbp* was expressed at similar levels in swarm and swim cells; the ratio of expression between the two states was 1.2 (Fig. 3b). *cysK* was expressed at a level 68-fold greater in the swarm state. In this state all operons in the CysB regulon were induced between 14- and 68-fold (Fig. 3).

To test whether environmental sulfur availability affected induction of the CysB regulon in swarm cells, *cysJ* expression was used to represent CysB regulon activity, as the cells were grown in NBG swarm and swim media

Fig. 3. (a) Photographs of *S. typhimurium* cultures with *cys* promoter transcriptional fusions to *luxCDABE* on swim (left column) and swarm (right column) NBG plates (0.25 and 0.5% agar, respectively). Photographs were taken in the dark and represent endogenous light production. (b) Quantitative measurement of expression of *cys* genes and *sbp* in swarm or swim states, grown on NBG glucose. Black bars represent swim-cell values; white bars represent swarm-cell values. The results are means $\pm$ SE of three independent biological replicates. RLU are calculated from luciferase activity (c.p.s.) normalized to 10^7 c.f.u. ml $^{-1}$ (1 RLU=1 c.p.s. per 10^7 c.f.u.).

with sulfur sources added. There was no expression of *cysJ* in swim cells with any of the sulfur sources tested (Fig. 4). In the swarm state, *cysJ* expression was detected in unsupplemented medium and with all exogenous sulfur sources except cysteine. Growth on sulfite, thiosulfate or

Fig. 4. Expression of the *cysJIH* promoter transcriptional fusion to *luxCDABE* in response to varying sulfur sources added to NBG swarm medium. The final concentration of each sulfur source added to NBG was 0.5 mM, except for cysteine (0.2 mM). Black bars represent swim-cell values; white bars represent swarm-cell values. The *cysJIH* promoter is used as a representative of the CysB regulon. Error bars represent standard error from two biological replicates. RLU are calculated from luciferase activity (c.p.s.) normalized to 10^7 c.f.u. ml⁻¹.

sulfide reduced *cysJ* expression to a similar level. Expression of *cysJ* was the greatest on unsupplemented medium and medium supplemented with sulfate, which modestly reduced expression levels (2.5-fold).

1,2,4-Triazole can be used to produce swarm phenotypes of cysteine auxotrophs

Cysteine metabolism can also be studied by chemical genetics, which employs small molecules to inhibit the function of a gene product, and confer a phenotype corresponding to the inactivation of a particular gene. 1,2,4-Triazole induces cysteine auxotrophy in wild-type cells by reacting with OAS in a reaction catalysed by CysK to produce an alanine, rather than a cysteine, product (Hulanicka *et al.*, 1972).

The motility and antibiotic resistance phenotypes of wild-type *S. typhimurium* were examined in the presence of 1,2,4-triazole. At concentrations up to 100 mM, swim and swarm motility were unaffected (data not shown). In medium supplemented with 75 mM and 100 mM 1,2,4-triazole, the MIC of gentamicin decreased to 12 µg ml⁻¹ and 8 µg ml⁻¹, respectively, in swarm cells, from >256 µg ml⁻¹ in untreated swarm cells. These values are comparable to MICs observed for swim cells of either cysteine auxotrophs or wild-type cells. The MIC of ciprofloxacin dropped from 12 µg ml⁻¹ in untreated cells to 0.064 µg ml⁻¹ in medium containing 75 mM and 100 mM 1,2,4-triazole, similar to the level obtained with most cysteine auxotrophs on standard swarm medium.

Induction of the CysB regulon was estimated by measuring the amount of expression from the *cysJ* promoter-luciferase fusion. Addition of 1,2,4-triazole decreased *cysJ*

expression in a dose-dependent manner (Fig. 5). Compared to the standard swarm medium, *cysJ* expression decreased 4-fold and 7.5-fold on NBG supplemented with 5 mM or 10 mM 1,2,4-triazole, respectively. When cells were grown on NBG supplemented with 100 mM 1,2,4-triazole, *cysJ* expression was below detectable levels.

The use of 1,2,4-triazole to block cysteine biosynthesis confirms previous results obtained by *cys* gene disruption. These results indicated that the chemical induction of *cys* auxotrophy was comparable to genetic disruption of *cys* biosynthesis in preventing induction of antibiotic resistance in the swarm state.

Chemical composition of swarm medium

Since Nutrient Broth (NB) supported the growth of all cysteine auxotrophs, it is clear that the medium contains sufficient cysteine and reduced sulfur to support growth of *S. typhimurium*. However, actively migrating swarm cells induced the genes for cysteine biosynthesis, and cysteine biosynthesis is required for the antibiotic resistance phenotype. The concentration of cysteine in NB was determined by HPLC. DTT was added to reduce disulfide bonds between free cysteine and thiol groups in peptides, allowing the quantification of total free cysteine in the medium. The concentration of cysteine in NB and after reduction with DTT was found to be 5.0 ± 2.1 µM and 21.3 ± 1.5 µM, respectively.

NB containing oligopeptides may provide a source of cysteine to *S. typhimurium* and would not be reflected in our amino acid analysis. To measure the cysteine in peptides, HPLC analysis was repeated on NB digested with proteinase K. This increased concentrations of the most abundant amino acids; however, the concentration of cysteine increased only modestly to 29.0 ± 4.3 µM.

Cysteine at a concentration of 30 µM as the only sulfur source in minimal medium allowed weak growth of

Fig. 5. Effect of 1,2,4-triazole on expression of the *cysJIH* promoter transcriptional fusion to *luxCDABE* in swarm cells. Error bars represent standard error from at least two independent biological replicates. Relative light units (RLU) are calculated from luciferase activity (c.p.s.) normalized to 10^7 c.f.u. ml⁻¹.

wild-type and was insufficient to support growth of auxotrophs. In M9 minimal medium broth or agar plates, a *cysE* mutant requires 50 μ M cysteine to grow (data not shown).

From these results, it is evident that NB contains sufficient cysteine for growth but not enough for swarm-cell differentiation, either free or in peptides. Other reduced sulfur sources, such as thiosulfate or sulfide, may be present at a concentration to permit the production of cysteine without inducing the CysB regulon in wild-type swim cells.

DISCUSSION

Swarm cells have phenotypes reflecting a different cellular physiology that extend beyond swarm motility. These cells show elevated antibiotic resistance, increased capacity for cell–cell signalling, and a switch to an anabolic metabolism (Kim *et al.*, 2003; Kim & Surette, 2004, 2006). In this paper, we show that cysteine auxotrophs are impaired in the antibiotic resistance phenotype and that regulatory mutants of cysteine biosynthesis are incapable of swarm motility. All *cys* mutants in the sulfate assimilation pathway were still capable of swarm motility; however, these auxotrophs did not exhibit the increased antibiotic resistance observed in wild-type swarm cells. These auxotrophs are probably still capable of synthesizing cysteine from reduced sulfur sources in the medium, since CysK is expressed abundantly at basal levels without CysB such that sulfide can support the growth of a *cysB* strain (Kredich, 1971).

The only mutants that were incapable of swarming on the standard swarm medium were the regulatory mutants *cysB* and *cysE*. To our knowledge, this is the first report of a cysteine auxotroph that is unable to swarm. Previously, mutants in the histidine biosynthetic pathway were identified in a screen of mutants unable to swarm on Difco agar; however, these mutants were not investigated further (Toguchi *et al.*, 2000). Other screens for swarm-defective mutants in *S. typhimurium* identified genes in LPS biosynthesis and chemotaxis (Harshey & Matsuyama, 1994; Kim *et al.*, 2003; Toguchi *et al.*, 2000). Induction of the *cys* biosynthetic genes would not be possible in either *cysB* or *cysE* mutants, indicating that these mutants would rely on imported cysteine for growth (Baptist & Kredich, 1977). Another strain entirely reliant on imported cysteine for survival, a *cysK cysM* double mutant, is still capable of swarming. One difference between the biosynthetic mutants and the regulatory mutants lies in the CTS-1 cysteine permease that is induced by CysB. CTS-1 has the highest capacity for cysteine of the three cysteine permeases and is thought to be responsible for rapid cysteine transport during times of cysteine starvation (Baptist & Kredich, 1977). Interestingly, *cysB* and *cysE* are still motile and are not impaired in swimming or chemotaxis behaviour. Thus, the two lower-capacity cysteine permeases, CTS-2 and CTS-3, may fulfil the cysteine requirements of swim cells but not swarm cells.

An increased requirement for cysteine in swarm cells may explain why cells grown on swim or swarm plates have different profiles of *cys* gene expression. Swim cells have no detectable expression of cysteine biosynthetic genes except for *cysK* and *sbp*. In *Escherichia coli*, *sbp* is positively regulated by Cbl, a transcriptional regulator with 60% amino acid similarity to CysB, involved in the regulation of cysteine biosynthesis from organic sulfur sources (Kredich, 1996; Stec *et al.*, 2006; Van Der Ploeg *et al.*, 1997). *S. typhimurium* does not contain a Cbl homologue. *cysK* has a significant level of basal expression in the absence of CysB activation as observed in swim cells. All other *cys* operons had very low activity on swim plates and were induced in swarm conditions, consistent with CysB activation.

Interestingly, *cysE* expression was also induced in the swarm state. Transcriptional regulation of *cysE* is not known to occur and it is not thought to be part of the CysB regulon (Kredich, 1996). In *E. coli*, serine acetyltransferase is implicated in having a role in biofilm formation since *cysE* mutants formed biofilms more quickly than wild-type cells (Sturgill *et al.*, 2004). The authors postulated that serine acetyltransferase may be producing an extracellular signalling molecule that is neither OAS nor NAS. Unexpectedly, the addition of cysteine decreased the rate of biofilm formation. If CysE produces an extracellular signal required for swarm motility, it is not likely to account for why Δ *cysE* did not swarm unless cysteine was provided since the Δ *cysB* strain also did not swarm, and in this strain serine acetyltransferase and OAS would be produced.

CysB is known to activate transcription of genes that do not have a direct role in cysteine biosynthesis. Arginine decarboxylase expression is increased by CysB binding its promoter, and *cysB* mutants do not ferment many carbohydrates as well as the parent strains of *E. coli* and *S. typhimurium* (Quan *et al.*, 2002; Shi & Bennett, 1994). Since CysB is the regulator of sulfur metabolism in the cells, a central metabolic pathway, it could be considered a global regulator in *S. typhimurium*. Cross-talk between metabolic pathways could help the bacterium coordinate utilization of a metabolite in one metabolic pathway with those in another (Quan *et al.*, 2002). In addition, *cysB* and *cysE* mutants of *S. typhimurium* and *E. coli* have been reported to have increased resistance to the β -lactam antibiotic mecillinam and the quinolone novobiocin (Anton, 2000; Costa & Anton, 2006; Lilic *et al.*, 2003; Oppezzo & Anton, 1995; Rakonjac *et al.*, 1991). Since swarming motility in both *cysB* and *cysE* is restored by the inclusion of cysteine in the swarm medium, a CysB-regulated gene outside of the cysteine biosynthetic genes is not the reason that these strains are incapable of swarming on standard swarm medium. A *cysB*, but not a *cysE* strain can swarm in media supplemented with sulfide, indicating that sulfide is also limiting in swarm medium (data not shown). Exogenous cysteine also restores the antibiotic resistance phenotype, showing that cysteine and not those genes outside of cysteine biosynthesis in the CysB regulon are important.

It is apparent that differentiated swarm cells either cannot access the cysteine in NB or require more cysteine than swim cells. Swarm cells may be using the reduced sulfur for reasons other than growth, to maintain disulfide bond formation in extracellular and cytosolic proteins by the synthesis of glutathione and other cellular reductants (Bjur *et al.*, 2006). The sulfur in cysteine is used as the reduced sulfur source for the synthesis of sulfur-containing molecules in the cells in *S. typhimurium*. Swarm cells, by relying on oxidative phosphorylation for energy generation more than swim cells, may generate more reactive oxygen species (González-Flecha & Demple, 1995; Kim & Surette, 2004). This may induce oxidative stress pathways to prevent damage to cytoplasmic proteins. The glutathione- and thioredoxin-reduction systems are critical for maintaining the reduction potential of the cytoplasm; these reductants and their respective reductases in most cases contain cysteine residues (Carmel-Harel & Storz, 2000). Thus, cysteine has a central role in maintaining the reducing environment of the cytoplasm. If *cys* auxotrophs are oxidatively stressed, the added stress of an antibiotic may lower the MIC for a particular antibiotic. Alternatively, an antibiotic may cause further oxidative stress and if auxotrophs have impaired responses to oxidative stress, they may have a lower MIC. Variable *soxS* transcript levels were observed in efflux pump mutants of *S. typhimurium* (Eaves *et al.*, 2004).

This research indicates an important role for cysteine in *S. typhimurium* swarm cells, as cysteine biosynthetic regulatory mutants fail to swarm, cysteine auxotrophs exhibit decreased MICs for two unrelated antibiotics in the swarm state, and wild-type cells induce the cysteine biosynthetic operon in the swarm state. Use of chemical genetics to target the cysteine biosynthetic pathway and reduced antibiotic resistance in the swarm state provides potential for therapeutic use of compounds to prevent the induction of intrinsic antibiotic resistance pathways. Our current hypothesis is that swarm cells require additional cysteine for reasons other than growth, perhaps to counter oxidative stress generated by oxidative phosphorylation. We are currently testing this hypothesis.

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REFERENCES

- Allison, C., Lai, H. C. & Hughes, C. (1992a). Co-ordinate expression of virulence genes during swarm-cell differentiation and population migration of *Proteus mirabilis*. *Mol Microbiol* **6**, 1583–1591.
- Allison, C., Coleman, N., Jones, P. L. & Hughes, C. (1992b). Ability of *Proteus mirabilis* to invade human urothelial cells is coupled to motility and swarming differentiation. *Infect Immun* **60**, 4740–4746.
- Anton, D. N. (2000). Induction of the cysteine regulon of *Salmonella typhimurium* in LB medium affects the response of *cysB* mutants to mecillinam. *Curr Microbiol* **40**, 72–77.
- Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L. & Leibler, S. (2004). Bacterial persistence as a phenotypic switch. *Science* **305**, 1622–1625.
- Baptist, E. W. & Kredich, N. M. (1977). Regulation of L-cysteine transport in *Salmonella typhimurium*. *J Bacteriol* **131**, 111–118.
- Bjarnason, J., Southward, C. M. & Surette, M. G. (2003). Genomic profiling of iron-responsive genes in *Salmonella enterica* serovar Typhimurium by high-throughput screening of a random promoter library. *J Bacteriol* **185**, 4973–4982.
- Bjur, E., Eriksson-Ygberg, S., Fredrik Aslund, F. & Rhen, M. (2006). Thioredoxin 1 promotes intracellular replication and virulence of *Salmonella enterica* serovar Typhimurium. *Infect Immun* **74**, 5140–5151.
- Burkart, M., Toguchi, A. & Harshey, R. M. (1998). The chemotaxis system, but not chemotaxis, is essential for swarming motility in *Escherichia coli*. *Proc Natl Acad Sci U S A* **95**, 2568–2573.
- Carmel-Harel, O. & Storz, G. (2000). Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. *Annu Rev Microbiol* **54**, 439–461.
- Costa, C. S. & Anton, D. N. (2006). High-level resistance to mecillinam produced by inactivation of soluble lytic transglycosylase in *Salmonella enterica* serovar Typhimurium. *FEMS Microbiol Lett* **256**, 311–317.
- 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.
- Eaves, D. J., Ricci, V. & Piddock, L. J. V. (2004). Expression of *acrB*, *acrF*, *acrD*, *marA*, and *soxS* in *Salmonella enterica* serovar Typhimurium: role in multiple antibiotic resistance. *Antimicrob Agents Chemother* **48**, 1145–1150.
- Fraser, G. M. & Hughes, C. (1999). Swarming motility. *Curr Opin Microbiol* **2**, 630–635.
- González-Flecha, B. & Demple, B. (1995). Metabolic sources of hydrogen peroxide in aerobically growing *Escherichia coli*. *J Biol Chem* **270**, 13681–13687.
- Harshey, R. M. (2003). Bacterial motility on a surface: many ways to a common goal. *Annu Rev Microbiol* **57**, 249–273.
- Harshey, R. M. & Matsuyama, T. (1994). Dimorphic transition in *Escherichia coli* and *Salmonella typhimurium*: surface-induced differentiation into hyperflagellate swarmer cells. *Proc Natl Acad Sci U S A* **91**, 8631–8635.
- Henrichsen, J. (1972). Bacterial surface translocation: a survey and a classification. *Bacteriol Rev* **36**, 478–503.
- Hryniewicz, M. M. & Kredich, N. M. (1991). The *cysP* promoter of *Salmonella typhimurium*: characterization of two binding sites for CysB protein, studies of in vivo transcription initiation, and demonstration of the anti-inducer effects of thiosulfate. *J Bacteriol* **173**, 5876–5886.
- Hulanicka, D., Klopotoski, T. & Smith, D. A. (1972). The effect of triazole on cysteine biosynthesis in *Salmonella typhimurium*. *J Gen Microbiol* **72**, 291–301.
- Kim, W. & Surette, M. G. (2003). Swarming populations of *Salmonella* represent a unique physiological state coupled to multiple mechanisms of antibiotic resistance. *Biol Proced Online* **5**, 189–196.

- Kim, W. & Surette, M. G. (2004). Metabolic differentiation in actively migrating swarming *Salmonella*. *Mol Microbiol* **54**, 702–714.
- Kim, W. & Surette, M. G. (2006). Coordinated regulation of two independent cell-cell signalling systems and swarmer differentiation in *Salmonella enterica* serovar Typhimurium. *J Bacteriol* **188**, 431–440.
- Kim, W., Killam, T., Sood, V. & Surette, M. G. (2003). Swarm-cell differentiation in *Salmonella enterica* serovar Typhimurium results in elevated resistance to multiple antibiotics. *J Bacteriol* **185**, 3111–3117.
- Kredich, N. M. (1971). Regulation of L-cysteine biosynthesis in *Salmonella typhimurium*. I. Effects of growth on varying sulfur sources and O-acetyl-L-serine on gene expression. *J Biol Chem* **246**, 3474–3484.
- Kredich, N. M. (1996). Biosynthesis of cysteine. In *Escherichia coli and Salmonella*, pp. 514–527. Edited by R. Curtiss, III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter & H. E. Umbarger. Washington: ASM Press.
- Levin, B. R. & Rozen, D. E. (2006). Non-inherited antibiotic resistance. *Nat Rev Microbiol* **4**, 556–562.
- Liaw, S.-J., Lai, H.-C., Ho, S.-W., Luh, K.-T. & Wang, W.-B. (2003). Role of RsmA in the regulation of swarming motility and virulence factor expression in *Proteus mirabilis*. *J Med Microbiol* **52**, 19–28.
- Lilic, M., Jovanovic, M., Jovanovic, G. & Savic, D. J. (2003). Identification of the CysB-regulated gene, *hslJ*, related to the *Escherichia coli* novobiocin resistance phenotype. *FEMS Microbiol Lett* **224**, 239–246.
- Maloy, S. R. (1990). Phage P22. In *Experimental Techniques in Bacterial Genetics*, pp. 11–16. Boston, MA: Jones & Bartlett Publishers.
- Neuwald, A. F., Krishnan, B. R., Brikun, I., Kulakauskas, S., Suziedelis, K., Tomcsanyi, T., Leyh, T. S. & Berg, D. E. (1992). *cysQ*, a gene needed for cysteine synthesis in *Escherichia coli* K-12 only during aerobic growth. *J Bacteriol* **174**, 415–425.
- Oppezzo, O. J. & Anton, D. N. (1995). Involvement of *cysB* and *cysE* genes in the sensitivity of *Salmonella typhimurium* to mecillinam. *J Bacteriol* **177**, 4524–4527.
- Ostrowski, J. & Kredich, N. M. (1990). In vitro interactions of CysB protein with the *cysJIIH* promoter of *Salmonella typhimurium*: inhibitory effects of sulfide. *J Bacteriol* **172**, 779–785.
- Peck, H. D. (1961). Enzymatic basis for assimilatory and dissimilatory sulfate reduction. *J Bacteriol* **82**, 933–939.
- Quan, J. A., Schneider, B. L., Paulsen, I. T., Yamada, M., Kredich, N. M. & Saier, M. H., Jr (2002). Regulation of carbon utilization by sulfur availability in *Escherichia coli* and *Salmonella typhimurium*. *Microbiology* **148**, 123–131.
- Rakonjac, J., Milic, M. & Savic, D. J. (1991). *cysB* and *cysE* mutants of *Escherichia coli* K12 show increased resistance to novobiocin. *Mol Gen Genet* **228**, 307–311.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. New York, NY: Cold Spring Harbor Laboratory Press.
- Shi, X. & Bennett, G. N. (1994). Effects of *rpoA* and *cysB* mutations on acid induction of biodegradative arginine decarboxylase in *Escherichia coli*. *J Bacteriol* **176**, 7017–7023.
- Sirko, A., Zatyka, M., Sadowy, E. & Hulanicka, D. (1995). Sulfate and thiosulfate transport in *Escherichia coli* K-12: evidence for a functional overlapping of sulfate- and thiosulfate-binding proteins. *J Bacteriol* **177**, 4134–4136.
- Stec, E., Witkowska-Zimny, M., Hryniewicz, M. M., Neumann, P., Wilkinson, A. J., Brzozowski, A. M., Verma, C. S., Zaim, J., Wysocki, S. & Bujacz, G. D. (2006). Structural basis of the sulphate starvation response in *Escherichia coli*: crystal structure and mutational analysis of the cofactor-binding domain of the Cbl transcriptional regulator. *J Mol Biol* **364**, 309–322.
- Sturgill, G., Toutain, C. M., Komperda, J., O'Toole, G. A. & Rather, P. N. (2004). Role of CysE in production of an extracellular signaling molecule in *Providencia stuartii* and *Escherichia coli*: loss of *cysE* enhances biofilm formation in *Escherichia coli*. *J Bacteriol* **186**, 7610–7617.
- Toguchi, A., Siano, M., Burkart, M. & Harshey, R. M. (2000). Genetics of swarming motility in *Salmonella enterica* serovar Typhimurium: critical role for lipopolysaccharide. *J Bacteriol* **182**, 6308–6321.
- Van Der Ploeg, J. R., Iwanicka-Nowicka, R., Kertesz, M. A., Leisinger, T. & Hryniewicz, M. M. (1997). Involvement of CysB and Cbl regulatory proteins in expression of the *tauABCD* operon and other sulfate starvation-inducible genes in *Escherichia coli*. *J Bacteriol* **179**, 7671–7678.

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