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Metabolic differentiation in actively swarming *Salmonella*

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Summary

Most current paradigms of microbial metabolism have been derived from studying cells grown under a variety of nutrient compositions in aqueous environments. With recent advances in genomics and experimental techniques, alternative forms of bacterial growth are increasingly being explored. When propagated on nutrient-rich semi-solid media, several species of bacteria undergo a morphological differentiation into swarmers that are capable of migrating on surfaces. Recent studies indicate that swarmer differentiation represents much more than a motility phenotype, as several clinically important attributes are also co-regulated. We demonstrate that migrating swarmer cells of *Salmonella* are metabolically differentiated compared to the vegetative swimmer cells grown in the same nutrient environment. Furthermore, once the cells have differentiated, the swarmers remain in this physiological state under conditions that do not promote the initial differentiation. The bacterium's capacity to override some of the classic paradigms of metabolic regulation established in aqueous environments represents a unique physiological response by the pathogen that may be advantageous in polymicrobial environments such as the host.

Introduction

Motility is one of the most extensively studied cellular processes in bacteria. Most studies focus on swimming in aqueous environments, but other motility behaviours are observed when bacteria are propagated outside an aqueous setting (Harshey, 2003). Swarming motility is a collective behaviour of groups of bacterial cells associated with migration on semi-solid surfaces (Fraser and Hughes, 1999). Unlike the classic swimming motility in aqueous environments, vegetative cells must first differentiate into elongated and hyperflagellated swarmer cells to migrate on the surface (Harshey and Matsuyama, 1994; Fraser and Hughes, 1999). Besides the obvious physical changes, swarmer differentiation can also be coupled to increased expression of important virulence determinants in some species (Fraser and Hughes, 1999).

Salmonella enterica serovar Typhimurium undergoes swarmer differentiation in semi-solid media [0.4–1.0% agar (w/v)], and remains undifferentiated when propagated in media with agar concentrations below or above this range (Harshey and Matsuyama, 1994; Kim and Surette, 2003). At present, the biological role of swarming motility in *Salmonella* remains to be elucidated. Swarming may be utilized by the uropathogen, *Proteus mirabilis*, to ascend the host's urinary tract to colonize the kidney (Fraser and Hughes, 1999). *Salmonella* may also utilize swarming to ascend the biliary tract in a similar fashion, which along with the gall bladder are the known reservoirs for chronic infection (Finlay, 1994; Prouty *et al*., 2002). Interestingly, a mutation that conditionally impairs swarming lies in the *shdA* locus (Toguchi *et al*., 2000), which encodes an adhesin recently implicated in the persistence of *Salmonella* in the intestinal tract (Kingsley *et al*., 2002).

We have recently observed that, in comparison to the vegetative cells, differentiated swarmers of serovar Typhimurium exhibit elevated resistance to a broad spectrum of antibiotics (Kim *et al*., 2003), and as they de-differentiate back into the vegetative state, antibiotic sensitivity is also restored (Kim and Surette, 2003). Consequently, cells that are propagated on solid media and swim media share identical antibiotic resistance profiles, suggesting that swarm cells are phenotypically distinct from the undifferentiated vegetative cells (Kim *et al*., 2003). Furthermore, elevated resistance of the swarmers to cationic anti-microbial compounds is intimately linked to increased expression of the *pmrHFIJKLM* operon in comparison to the vegetative cells, regardless of their propagation in swim or solid media (Kim *et al*., 2003; Kim and Surette, 2003). Such phenotypic differences suggest that populations of vegetative swimmers and swarmers may represent quite distinct physiological states, and the motility behaviour is just one of many phenotypic changes that are associated with swarmer differentiation.

To address the hypothesis that swarm cells represent a

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distinct state with adaptations in addition to the observed motility phenotype, we used a proteomic approach to compare the states of swimmer and swarmer cells. Although the nutrient medium differed only in the agar concentration for the two populations of cells, we observed a systemic shift in basic metabolism. The observed changes in cellular physiology can account for some of the observed adaptive antibiotic resistance. The differentiation of migrating swarm cells may be an adaptive response stimulated by the conditions in the host, which may aid in the proliferation and persistence of *Salmonella* within the competitive environment of the gastrointestinal tract.

Results and discussion

Growth comparison of swimmer and swarmer populations

Growth curves of the swimmer and swarmer populations of serovar Typhimurium were compared to identify their respective exponential phases, from which the cells were sampled and processed for proteomic analyses. Because a single swarmer cell can represent multiple units of vegetative swimmer cells and a swarming population comprises cells of various lengths (Fraser and Hughes, 1999), the growth rates of the two cell populations cannot be accurately compared by the conventional methods of plating out serial dilutions or measuring optical density. As an alternative method for monitoring growth, we utilized a fluorescent DNA stain to measure temporal changes in the total DNA concentrations of the two cell populations. In swarm media, active migration of the colony began 5–6 h after inoculation, and the swarmers exhibited exponential growth until the swarm-front reached the edges of the plate 3–4 h later (Fig. 1). Under our growth conditions, swarm migration was not associated with formation of swarm rings (Fig. 1A), which represent periodic de-differentiation and re-differentiation phases typically exhibited by swarming *Proteus* spp. (Fraser and Hughes, 1999). In contrast, swarming populations of serovar Typhimurium remained 'locked' in an exponential mode of growth (doubling time of 29.8 min) once the swarm migration initiated (Fig. 1B), and actively migrating swarmers occupied the entire surface of the plates within 3–4 h (Fig. 1A). The swimmer population grew exponentially between 3 and 5 h and exhibited a comparable growth rate (doubling time of 25.9 min) as the actively migrating swarmer population (Fig. 1B).

Proteomic analysis of swimmer and swarmer populations

We explored the physiological differences between two populations of serovar Typhimurium using a two-dimensional gel electrophoresis (2DGE)-based proteomic approach to compare whole-cell protein samples extracted from exponentially growing swimmer and swarmer populations. In total, 11 sets of two-dimensional gels (2D-gels) were compared, representing different extract samples, pH ranges in the first dimension and staining techniques. Representative sets of the 2D-gels are shown in Fig. 2. Although some variations were observed among individual sets of gels, only those protein spots that were detected to be consistently different between the two cellular states were declared to be differentially regulated. Over 130 protein spots were identified by MALDI-TOF MS and grouped according to common pathways or physiological functions in Table 1.

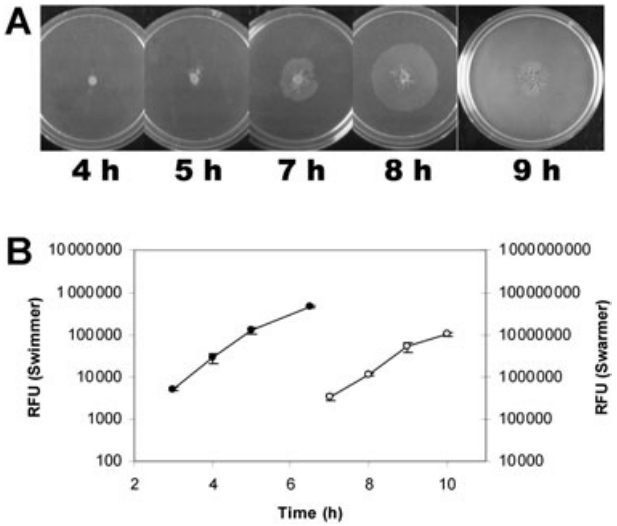


Fig. 1. Temporal images of active swarm migration (A) and growth profiles of swimmer and swarmer populations (B). The growth of the swimmers (●) and the swarmers (○) were monitored over time as a function of changing DNA concentration. DNA concentration is represented as relative fluorescence units (RFU), and the error bars represent the standard deviation.

Although the chemical composition of the nutrient medium differed only in the agar concentration for growing the two populations of cells, some fundamental physicochemical differences exist between the environments within which the two populations were propagated. Nonetheless, the proteomic analyses presented in this study are based on cells that were sampled from two populations exhibiting parallel balanced growth at similar replication rates (Fig. 1B) in the same nutrient environment. The majority of the differentially expressed proteins were identified to be associated with basic metabolism. While it is not possible to interpret the significance of fold-differences in the expression of individual proteins, many of the differentially expressed proteins cluster within common pathways and physiological functions that themselves are also interconnected.

Differentially regulated proteins involved in de novo biosynthetic pathways. One of the striking differences in the differentiated swarmers was the elevated expression of

enzymes involved in the *de novo* biosynthetic pathways. Numerous proteins associated with amino acid biosynthetic pathways (Bono *et al*., 1998) were upregulated. Chorismate (via AroA), carbamoyl phosphate (via CarA) and glutamine (via GlnA) serve as precursors of aromatic amino acids, arginine, glutamate and proline. AsnA and CysK are involved in the biosynthetic pathways of asparagine and serine respectively. In contrast, WraB was downregulated, which was previously shown to indirectly repress the transcription of the tryptophan biosynthetic genes by activating the repressor TrpR (Yang *et al*., 1993). Carbamoyl phosphate is also a key precursor in the biosynthesis of pyrimidine nucleotides (Neuhard and Kelln, 1996). In addition, the catalytic subunit (PyrB) of the aspartate carbamoyltransferase was also upregulated in the swarmers. PyrB performs the first enzymatic step in the *de novo* synthesis of pyrimidine nucleotides (Neuhard and Kelln, 1996). Elevated expression of the nucleoside diphosphate kinase (Ndk) in the swarmers suggests an increase in the intracellular levels and/or interconversion of (d)NTP (Neuhard and Kelln, 1996).

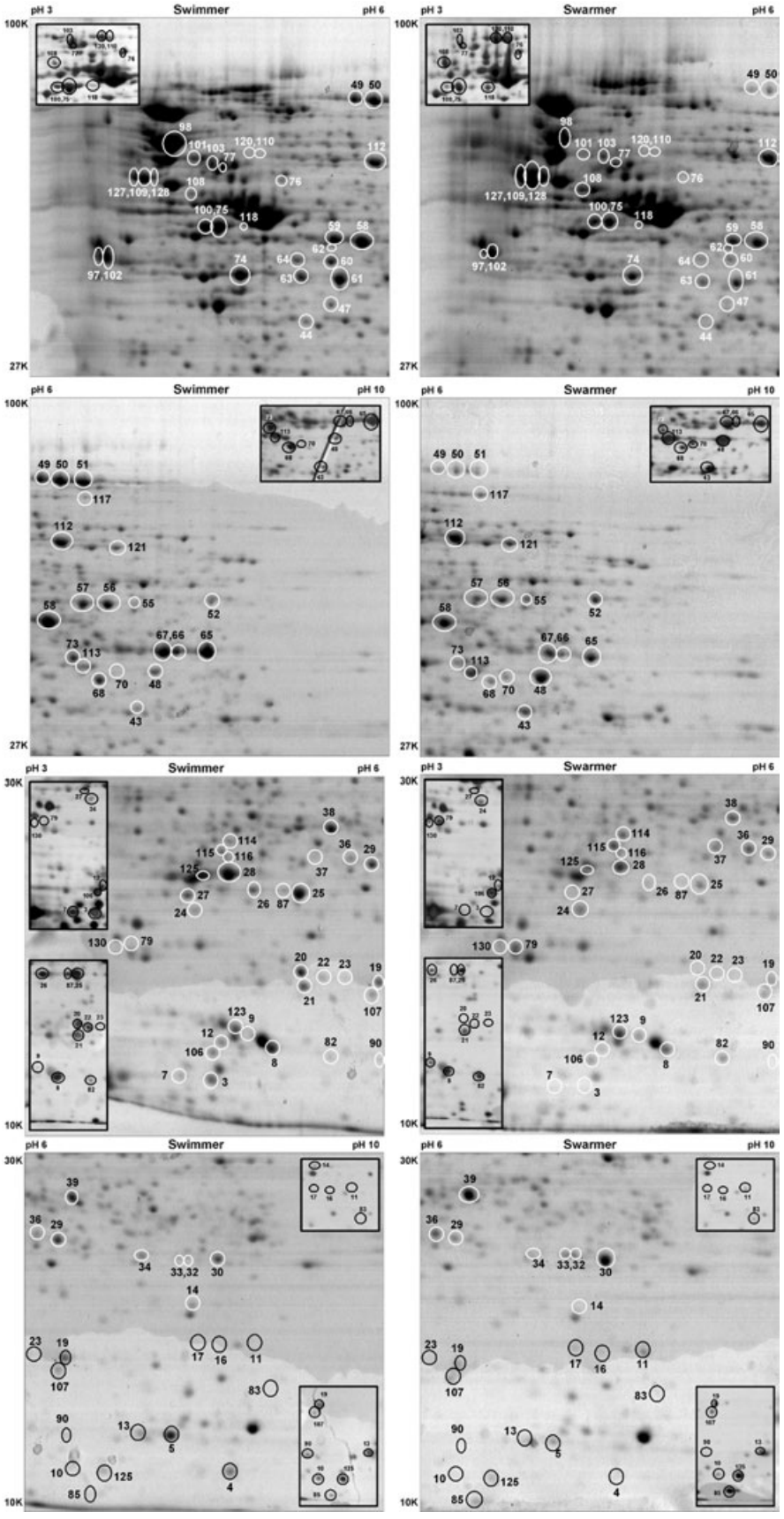
In contrast to the members of biosynthetic pathways, enzymes associated with the catabolism of exogenous peptide substrates and intracellular proteins, and nucleotide salvage pathways were downregulated in the swarmers. Furthermore, expression of several outer membrane porins was also decreased, including OmpA, which forms a non-specific diffusion channel for the uptake of various sugars and amino acids (Sugawara and Nikaido, 1992). Collectively, the data suggest that the differentiated swarm cells may preferentially synthesize these compounds *de novo*, rather than utilizing the exogenous substrates present in the medium, despite the fact that the cells are cultured in a nutrient-rich medium.

Consistent with increased expression of enzymes associated with *de novo* amino acid biosynthesis, proteins in the nitrogen assimilation pathways were also upregulated, including the regulatory protein GlnK and glutamine synthetase (GS). The bulk of cellular nitrogen is derived from ammonia assimilation, producing either glutamate (via glutamate dehydrogenase) or glutamine (via GS). In contrast to glutamate dehydrogenase, GS consumes ATP during assimilation; thus, it is preferentially used when ATP is readily available (Helling, 1998). Increased expression of GlnA and GlnK in a nitrogen-rich environment [i.e. glucose-supplemented NB (NBG)] indicates that the swarmers do not behave as expected from the established paradigm (Reitzer, 2003). Elevated *de novo* biosynthetic pathways may exert significant demands on the intracellular nitrogen supplies, stimulating the swarmers to actively assimilate ammonia by expending ATP.

Differentially regulated proteins involved in central metabolism and energy production. The differentiated swarmers appear to utilize distinct metabolic pathways to generate additional energy and precursor metabolites to support the various biosynthetic pathways. Swarmers may also be unique in their preference for glucose utilization, because the tricarboxylic acid (TCA) cycle enzymes were upregulated and the enzymes of glycolysis reduced. Elevated expression of LpdA and SdhA in particular suggests that the swarmers are preferentially utilizing the full TCA cycle. LpdA and SdhA are components of multiple subunit dehydrogenase complexes that recycle 2-ketoglutarate and succinate back into the cycle (Cunningham *et al*., 1998). ThiG and ThiE are members of the biosynthetic pathway for thiamin(e) pyrophosphate, which is essential for the decarboxylating activities of the dehydrogenase complexes (White and Spenser, 1996). *Escherichia coli* and *Salmonella* grown in the presence of abundant glucose in an aqueous medium generally do not utilize the full TCA cycle, because the majority of the cellular energy is derived directly from glycolysis (Cronan and LaPorte, 1996; Fraenkel, 1996). Instead, the TCA cycle branches into two terminal biosynthetic pathways to generate succinate and 2-ketoglutarate from oxaloacetate (Cronan and LaPorte, 1996). Swarmers, however, probably utilize the full TCA cycle while growing exponentially in the presence of glucose; and rather than breaking down excess glucose, glycolysis may be more precisely tuned to meet the cellular demand for pyruvate and acetyl-CoA. The glycolytic enzymes may be reduced not because the swarmers are utilizing other carbon sources but because the use of the full TCA cycle permits more efficient use of glucose. In addition, given that the most significant metabolic pathway for producing CO_2 is the TCA cycle (Takayama *et al*., 1994), elevated expression of the recently characterized carbonic anhydrase, YadF (Hashimoto and Kato, 2003), would also limit the loss of carbon molecules in the form of CO_2 molecules to the atmosphere.

Intriguingly, a pair of isozymes of glycolysis was differentially regulated in the two cell populations. GpmA and PmgI are phosphoglycerate mutases that perform the interconversion of 2-phosphoglycerate and 3-phosphoglycerate in the glycolytic and gluconeogenic pathways (Fraser *et al*., 1999). In *E. coli*, both mutases are expressed in similar quantities, but GpmA is believed to be the dominant mutase as it exhibits greater than 10-

Fig. 2. Proteomic comparison of the swimmer and swarmer populations of *Salmonella enterica* serovar Typhimurium 14028 by 2DGE. Each of the large panels represents a quarter section of a colloidal Coomassie-stained image as indicated by the approximate pH and Mw ranges. The internal silver-stained images show the differential regulation of the less abundant proteins and the spot numbers correspond to the spots within the larger image. Individual protein spots are numbered as summarized in Table 1.


pH 3
Swimmer
pH 6
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27K
49 50
98
101
103
120,110
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127,109,128
108
76
100,75
118
59
58
62
60
74
64
63
61
97,102
47
44
pH 3
Swarmer
pH 6
100K
27K
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101
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120,110
77
112
127,109,128
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Swimmer
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107
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pH 6
Swarmer
pH 10
30K
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85
4

Table 1. Differentially regulated proteins between the swarmer and swimmer populations of *Salmonella enterica* serovar Typhimurium 14028.

Cellular function	Protein	Relative expression[a]	Spot No.[b]	Protein function[c,d]
Glycolysis	PfkA	– (3.0–4.9)	60, 64	6-Phosphofructokinase I
	Fba	– (2.0–3.5)	58, 59	Fructose-bisphosphate aldolase
	TpiA	– (2.1–3.7)	38	Triosephosphate isomerase
	GapA	– (1.5–6.4)	61, 63, 65, 66, 67, 73	Glyceraldehyde-3-phosphate dehydrogenase A
	Pgk	– (1.9–4.5)	75, 100	Phosphoglycerate kinase
	PmgI	– (2.9–6.1)	101	Phosphoglyceromutase 2
	GmpA	+ (1.7–3.3)	39	Phosphoglyceromutase 1
	ManX	– (3.5–3.6)	68	Mannose-specific enzyme IIAB
Pyruvate dissimilation	LpdA	+ (2.6–5.3)	121	Lipoamide dehydrogenase, pyruvate dehydrogenase complex
	PflB	– (2.3–12.2)	49, 50, 51	Pyruvate formate lyase I
	YfiD	– (5.7–27.2)	3, 7	Formate acetyltransferase
	AckA	– (1.7–4.2)	56, 57	Acetate kinase A
	HycA	– (2.7–6.4)	23	Transcriptional repressor of hyc and hyp operons
TCA cycle	IcdA	+ (2.1–6.7)	108	Isocitrate dehydrogenase
	LpdA	+ (2.6–5.3)	121	Lipoamide dehydrogenase, 2-ketoglutarate dehydrogenase complex
	SucC	+ (3.2–5.2)	118	Succinyl-CoA synthetase (β-subunit)
	SucD	+ (4.3–7.8)	43	Succinyl-CoA synthetase (α-subunit)
	SdhA	+ (4.9–5.8)	117	Succinate dehydrogenase, flavoprotein subunit
	Mdh	+ (4.3–4.7)	48	Malate dehydrogenase
	AspA	– (3.2–4.7)	103	Aspartate ammonia-lyase (aspartase)
Cofactors and energy production	ThiE	+ (2.6–5.0)	115	Thiamin phosphate pyrophosphorylase
	ThiG	+ (2.2–5.0)	114	Thiazole biosynthesis protein
	RibH	+ (1.5–2.9)	106	Riboflavin synthase (β-chain), FMN/FAD precursor
	NuoI	– (2.6–8.1)	87	NADH dehydrogenase I (chain I)
	YfhP	+ (2.7–10.3)	11, 16, 17	Potentially involved in assembly of Fe–S clusters
	AtpA	+ (1.6–2.5)	112	Membrane-bound ATP synthase, F1 sector (α-subunit)
	AtpC	+ (2.0–2.4)	125	Membrane-bound ATP synthase, F1 sector (ϵ-subunit)
	YadF	+ (1.7–7.9)	36, 37	Carbonic anhydrase
Nitrogen assimilation	GlnA	+ (2.2–8.0)	110, 120	Glutamine synthetase
	GlnK	+ (4.0–6.5)	85	Regulatory protein, P-II 2
Amino acids biosynthesis	AroA	+ (1.8–2.5)	52	3-Enolpyruvylshikimate-5-phosphate synthetase, chorismate synthesis
	AsnA	+ (2.7–10.1)	62	Asparagine synthetase A
	CysK	+ (2.5–3.3)	113	O-acetylserine sulphydrolase A (cysteine synthase A subunit)
	CarA	+ (5.3–6.0)	55	Carbamoyl-phosphate synthetase (glutamine-hydrolysing small subunit)
	GlnA	+ (2.2–8.0)	110, 120	Glutamine synthetase
	AspA	– (3.2–4.7)	103	Aspartate ammonia-lyase (aspartase)
	PepD	– (2.8–4.6)	77	Aminoacyl-histidine dipeptidase
	WraB	– (3.8–3.9)	29	trp-repressor binding protein
Purine and pyrimidine nucleotides biosynthesis	CarA	+ (5.3–6.0)	55	Carbamoyl-phosphate synthetase (glutamine-hydrolysing small subunit)
	PyrB	+ (3.6–6.5)	70	Aspartate carbamoyltransferase (catalytic subunit)
	Cdd	– (1.6–6.1)	47	Cytidine/deoxycytidine deaminase
	Hpt	– (1.7–4.0)	8	Hypoxanthine phosphoribosyltransferase
	Gpt	+ (2.3–2.5)	107	Guanine-hypoxanthine phosphoribosyltransferase
	Ndk	+ (2.8–4.3)	82	Nucleoside diphosphate kinase
Fatty acid and LPS biosynthesis	AccB	+ (4.3–9.2)	79, 130	Acetyl-CoA carboxylase (BCCP subunit), carrier of biotin
	RfbC	+ (3.0–3.9)	24	dTDP-4, deoxyrhamnose 3,5 epimerase, O-antigen biosynthesis
Protein folding, degradation, and ribosome-associated	MopA (GroEL)	– (1.8–5.3)	98	Chaperone Hsp60 with peptide-dependent ATPase activity
	MopB (GroES)	– (4.0–4.6)	12	Chaperone Hsp10
	STM1251	– (5.6–10.7)	20	Putative molecular chaperone (small heat shock protein)
	HslU	– (3.4–4.8)	76	ATPase component of the HslUV protease
	PpiB	– (2.5–3.7)	21	Peptidyl-prolyl *cis-trans* isomerase B (rotamase B)
	YfiA	– (3.9–6.5)	4	Ribosome associated factor, stabilizes ribosomes against dissociation
	RpsF	+ (1.6–2.0)	123	30S ribosomal (S6 subunit)
Detoxification of reactive oxygen species and DNA protection	SodA	+ (2.1–4.6)	30, 32, 33	Superoxide dismutase (manganese)
	SodB	– (3.2–6.5)	25, 26	Superoxide dismutase (iron)
	STM2446	– (2.6–5.2)	44	Putative iron-dependent peroxidase

Table 1. *cont.*

Cellular function	Protein	Relative expression[a]	Spot No.[b]	Protein function[c,d]
	STM0402	– (1.9–3.0)	28, 124	Putative thiol-alkyl hydroperoxide reductase
	Gst	– (2.8–5.1)	34	Glutathionine S-transferase
	YliJ	+ (3.3–4.8)	116	Putative glutathione S-transferase
	YbdQ (UspG)	– (1.6–2.8)	5, 13	Universal stress protein G, ATP-binding; protection against respiratory uncouplers
	Dps	– (2.4–8.1)	19, 22	Stress response DNA-binding protein, resistance to H_2O_2
	YecG (UspC)	– (2.4–6.2)	90	Universal stress protein C, UspA family; DNA protection
Structural components of the outer membrane	FliC	+ (2.3–4.7)	109, 127, 128	Flagellin, filament structural protein, flagellar biosynthesis
	OmpA	– (2.1–3.9)	74	Outer membrane porin, putative hydrogenase
	OmpD (NmpC)	– (1.4–4.7)	97, 102	Most abundant outer membrane porin
	OmpW	– (2.3–7.3)	27	Outer membrane protein, colicin S4 receptor; putative transporter
Unknown	STM1078	– (4.1–4.3)	10	Putative cytoplasmic protein
	YcfP	– (4.0–4.1)	14	Putative esterase
	YiaC	+ (7.0–8.7)	9	Putative acetyltransferase
	YigI	+ (3.6–4.9)	83	Putative protein PaaI, possibly involved in aromatic compounds catabolism

a. + indicates that the protein spot was upregulated in the swarmers and – indicates downregulation. Values within the parentheses represent the range of differential expression observed among the 11 sets of 2D-gels, indicating (minimum difference–maximum difference).
b. Protein spot numbers as shown in Fig. 2. In some instances, spots with distinct isoelectric points were identified as the same protein. Multiple spots are probably indicative of post-translational modifications, the identities of which cannot be conclusively deduced simply based on observed shifts in the isoelectric point.
c. Protein functions are based on McClelland *et al.* (2001), and additional references are indicated in the text.
d. We applied stringent criteria at both stages of declaring and identifying the differentially expressed protein species. Numerous proteins were excluded from this table because they did not meet the stringencies of either criteria. Some of those that were tentatively identified to be upregulated in the swarmers included AroK, HisD, DapA, GlyA, TyrB, PyrG, ApbE, GuaB, RfbK, LuxS and YhbN, and downregulated were Udp, RpiA, FliI, SlyA and OsmY. These expression changes are consistent with the metabolic differentiation described in the text; however, they are not discussed.

fold higher specific activity for reactions representing both directions of glycolysis and gluconeogenesis (Fraser *et al.*, 1999). Because the specific activity of PmgI is much lower, its activity may be sufficient when the upstream glycolytic enzymes are present in abundance. In contrast, elevated expression of GpmA in the swarmers may ensure flux through glycolysis at relatively lower levels of intermediates.

Distinct enzymes associated with the interconversion of pyruvate and acetyl-CoA were also differentially regulated. In addition to the 2-ketoglutarate dehydrogenase complex, LpdA is also a member of the pyruvate dehydrogenase complex which converts pyruvate into acetyl-CoA and CO_2 (Guest *et al.*, 1989). In contrast, two pyruvate formate lyases (PflB and YfiD) that convert pyruvate into acetyl-CoA and formate were downregulated in the swarmers. Acetate kinase (AckA) was also downregulated, which is involved in the pathway of interconverting acetyl-CoA into acetate (Pruss and Wolfe, 1994). These results collectively suggest that swimmers may secrete relatively greater amounts of formate and acetate into the extracellular milieu as by-products of glycolysis. The coupling of rapid dissimilation of glucose and an inefficient TCA cycle results in the excretion of large amount of acetate into the medium (Cronan and LaPorte, 1996; Huisman *et al.*, 1996). These predictions are in agreement with the previous observation that the pH of the growth medium remains neutral within a migrating swarming colony (Toguchi *et al.*, 2000), while the swimmer medium decreases in pH during growth, regardless of varying aeration (data not shown).

Elevated expression of numerous molecular chaperones and proteins associated with oxidative stress in the swimmer population may be an indirect metabolic consequence of secreting acidic intermediates into the growth medium. When adapted to mild acidic conditions, serovar Typhimurium exhibits increased tolerance towards a variety of environmental stresses, including reactive oxygen species (ROS) and heat (Leyer and Johnson, 1993). ROS production is greatly augmented by the presence of iron, and the solubility of intracellular iron increases inversely proportional to the intracellular pH (Hall and Foster, 1996). Clearly, the cells are well equipped to protect themselves from the detrimental effects of acid accumulation by expressing a battery of stress-relieving proteins.

Several enzymes associated with energy production were upregulated in the swarmers, including the riboflavin synthase RibH, and YfhP, which is believed to be involved in the assembly of iron–sulphur (Fe–S) clusters. Riboflavin is the precursor for the flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Bacher *et al.*, 1996), and along with Fe–S clusters (Frazzon *et al.*, 2002), represent components of key respiratory enzymes. In addition, elevated expression of the ATP synthase subunits may further supplement the necessary energy to support

the augmented biosynthetic pathways in the swarmers. Although these results may suggest that ATP production should be elevated in the swarmers, there was no significant difference in the intracellular ATP levels of the two populations [swimmers (0.34 ± 0.06 RLU); swarmers (0.43 ± 0.12 RLU)]. This is also in agreement with the accepted paradigm that constant energy charge is maintained despite rather dramatic changes in environmental conditions (Neidhardt *et al*., 1990).

Proteins associated with the cell envelope. Several structural proteins associated with the cell wall were also differentially regulated between the two populations. An enzyme in the O-antigen biosynthesis pathway and the major structural component of the flagella (FliC) were upregulated in the swarmers, reflecting the morphological changes associated with swarmer differentiation (Fraser and Hughes, 1999; Toguchi *et al*., 2000; Harshey, 2003). Conversely, the expression of several outer membrane proteins was downregulated, indicating that the outer membrane of the swarmers may be relatively less permeable. OmpA and OmpD are major porin proteins in serovar Typhimurium (Lee and Schnaitman, 1980). OmpA forms a non-specific diffusion channel that allows the outer membrane penetration of various solutes, including arabinose, glucose, disaccharides, amino acids and dipeptides (Sugawara and Nikaido, 1992). OmpW is a minor outer membrane protein that serves as the receptor for colicin S4, whereas its potential role as an amino acid transporter remains somewhat elusive (Pilsl *et al*., 1999).

Separating the nutrient requirements for swarmer differentiation and maintenance of the swarm state

Salmonella generally requires a rich semi-solid medium supplemented with an energy-rich carbon source, such as glucose, for swarmer differentiation (Harshey and Matsuyama, 1994; Kim *et al*., 2003). When propagated in semi-solid M9 minimal medium supplemented with either glucose (M9G) or glucose and casamino acids (M9GC), vegetative cells do not undergo swarmer differentiation (Fig. 3A). Similarly, swarming is not promoted on semi-solid LB unless glucose is provided (Harshey and Matsuyama, 1994) (Fig. 3A). In contrast, nutrient broth (NB) may contain enough basal nutrients and carbohydrates because it does not require any additional carbon source, but swarming occurs at a significantly reduced rate compared with NBG (Fig. 3A). These observations indicate that glucose metabolism provides a significant amount of energy during the initial differentiation process.

Before the event in which the differentiated swarmers actively migrate out from the site of inoculation, these cells are exposed to acidic pH; however, the pH remains neutral throughout the spatial zones occupied by actively swarming cells (Toguchi *et al*., 2000). These observations suggest that the actively swarming population are metabolically distinct from those that are initially restricted to the site of inoculation. If swarmer differentiation is truly coupled to reduced outer membrane permeability, enhanced *de novo* biosynthetic pathways and a shift in glucose utilization, differentiated swarmers should be able to proliferate independently, regardless of the nutrient composition of the growth medium, provided glucose is present. To test this hypothesis, hybrid semi-solid media were prepared, composed of NBG in the centre of the plate encircled with LB, LBG or M9G. Cells were inoculated into the NBG portion of the plates to promote differentiation, and the migration patterns were recorded 24 h later. The differentiated swarmers maintained their ability to migrate in M9G and LBG, but not in LB (Fig. 3B). Efficient utilization of the full TCA cycle in the presence of glucose and the enhanced *de novo* biosynthetic pathways

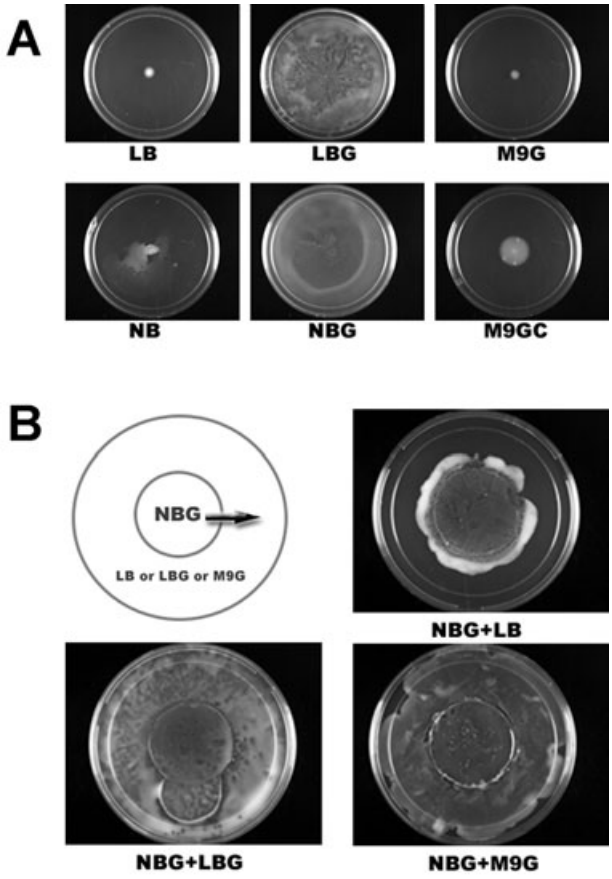

Fig. 3. Separating the nutrient requirements for initiating and maintaining the metabolic differentiation.
A. Swarming in different semi-solid media: LB, Luria broth; LBG, LB supplemented with 0.5% glucose; M9G, M9 supplemented with 0.5% glucose; M9GC, M9G supplemented with 0.1% casamino acids; NB, nutrient broth; NBG, NB supplemented with 0.5% glucose.
B. Swarming in hybrid media. The schematic illustrates the components of the hybrid system.

would allow the cells to maintain the swarm state in M9G. In contrast, growth in LB without the supplementation of glucose would ultimately result in reduced flow of phosphoenolpyruvate and pyruvate into the TCA cycle, consequently disrupting the metabolic circuit as the biosynthetic pathways cannot be fuelled with sufficient levels of precursor metabolites, reducing power and energy. Accordingly, swarming is halted shortly after the cells migrate into LB. The fact that the pre-differentiated swarmers maintained their ability to migrate in M9G, but not in LB, rules out the possibility that their ability to maintain swarming in M9G resulted simply from diffusion of nutrients (i.e. glucose) from the NBG portion of the hybrid media or previously synthesized extracellular polysaccharide slime associated with swarmer differentiation (Toguchi *et al*., 2000). The metabolically differentiated swarmers no longer require the nutrients that are essential for the initial differentiation of vegetative cells to maintain the swarm state. These results strongly support the proteomics data presented above, and are in agreement with the previous observations that mutations in genes that encode members of the PTS and *de novo* biosynthetic pathways conditionally disrupt swarming (Toguchi *et al*., 2000).

Comparison of spread-plated and actively migrating swarmers

In a recent microarray study, nearly one-third of the serovar Typhimurium genome was reported to be differentially regulated between cells grown on the surface and in broth, where genes associated with iron metabolism and several biosynthetic pathways were swarming specific (Wang *et al*., 2004). The swarm-population described by Wang *et al*. (2004) is fundamentally distinct from that described here because they extracted mRNA from cells that were spread-plated on the swarm medium. Although the spread-plated cells undergo swarmer differentiation, active migration is limited because the surface of the medium is crowded by neighbouring cells. In addition, because swarmer differentiation and migration is temporally dependent on the cellular density of the inoculum (Fraser and Hughes, 1999; Harshey, 2003), spread-plating the inoculum leads to distinct clusters of cells with varying density, promoting heterogenous populations at distinct stages of swarm differentiation. Actively migrating swarmers are exposed to a fundamentally distinct environment than those spread-plated, because the cells are inoculated in the centre of the swarm plates and the differentiated swarmers rapidly and uniformly migrate out into a fresh environment (Fig. 1). Our study has specifically addressed the physiological state of actively migrating swarm cells which exhibit unique phenotypes such as adaptive antibiotic resistance (Kim and Surette, 2003; Kim *et al*., 2003).

To ascertain whether these two approaches of propagating swarm cells potentially generate distinct swarm-populations, we utilized luciferase-based fusions to compare the transcriptional activities of genes representing glycolysis and the TCA cycle. The *pykF* gene encodes pyruvate kinase I, which carries out the interconversion of phosphoenolpyruvate and pyruvate (Fraenkel, 1996). Fumarase A, encoded by *fumA*, catalyses the interconversion of fumarate and malate in the TCA cycle (Cronan and LaPorte, 1996). Transcriptional activities were measured in cells harvested from two spatially distinct locations in the actively migrating colony: near the site of initial migration and at the edges of the swarm-front. The expression profiles of both genes were virtually identical between the two extreme spatial locations within the actively migrating colonies (Fig. 4A), supporting the notion that the actively swarming population is relatively homo-

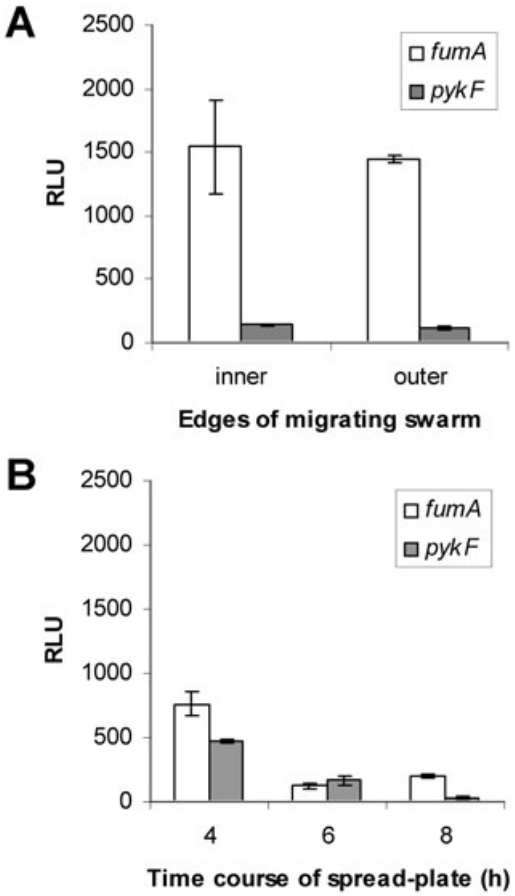

Fig. 4. luciferase-derived expression profiles of *fumA* and *pykF* genes in actively swarming and spread-plated populations in the NBG swarm medium.
A. Spatial expression profiles in cells from the inner and outer edges of actively migrating swarmers at 9 h.
B. Temporal expression profiles in cells spread-plated on the same swarm medium. Expression was normalized with the DNA concentration of each sample (space or time) and represented as relative light units (RLU), and the error bars represent the standard deviation.

geneous. In contrast, both genes exhibited temporal variations in transcriptional activity in the spread-plated populations (Fig. 4B). The relative ratios of *fumA* and *pykF* expression (i.e. TCA cycle to glycolysis) were significantly lower in the spread-plated populations in comparison to the actively migrating swarmers in the same swarm medium.

When we compare our proteomic data with the microarray data generated by Wang *et al.* (2004), there is no clear consensus. Numerous proteins/genes show opposite trends whereas some components of the data overlap between the two studies that are rather scattered among different pathways and physiological functions. One of the major differences is that some, but not all, of the members of the TCA cycle and glycolysis were upregulated in the microarray data set. Given the different populations of cells examined in the two studies, these reports do not necessarily contradict one another but represent complementary studies.

Adaptive antibiotic resistance and relevance of the swarm state in the host

A swarming population of *Salmonella* exhibits elevated resistance to a broad spectrum of anti-microbial compounds in comparison to vegetative cells propagated in swim (0.25% agar) or solid (1.5% agar) media (Kim *et al.*, 2003). Identical resistance profiles were observed in the vegetative populations regardless of the relative solidity of the media, which suggest that the diffusion rates of physically distinct anti-microbial compounds do not differ significantly within these media. Moreover, the metabolic shift in the swarmers does not simply result from differences in the diffusion rates of nutrients associated with surface growth in nutrient-rich NBG.

The multiple antibiotic resistance phenotype associated with the swarmers may be a corollary of reduced outer membrane permeability. In combination with increased positive charge of the outer membrane (Kim *et al.*, 2003; Kim and Surette, 2003), decreased expression of the porins may significantly reduce the entry of the antibiotics. In addition to synthetic anti-microbial compounds, reduced permeability may also provide cross-protection against the bactericidal toxins produced by the host and competing bacteria. *E. coli*-produced bacteriocins are active against closely related species including *Salmonella* (Braun *et al.*, 1994). Thus, reduced expression of the colicin receptor protein, OmpW, would confer an additional competitive advantage in the host's polymicrobic environment.

Metabolic differentiation in swarming serovar Typhimurium

The swarmer population of serovar Typhimurium represents a unique physiological state that is exemplified by its unconventional utilization of the classic metabolic pathways. A consistent trend was observed in the differentially regulated proteins between the swarmer and swimmer populations, in that the majority of the proteins within the common pathways were co-regulated. As summarized in Fig. 5, the differentially regulated proteins in the swarmers indicate that there is a systemic shift in basic metabolism. In comparison to its vegetative swimmer counterpart grown in the same nutrient-rich medium, the core difference with the swarmer population may lie in the coupling of elevated *de novo* biosynthetic pathways and relatively reduced outer membrane permeability. Reduced permeability of the outer membrane of the swarmers would also reduce the uptake rate of exogenous nutrients, and may ultimately exert more demand on the *de novo* biosynthetic pathways.

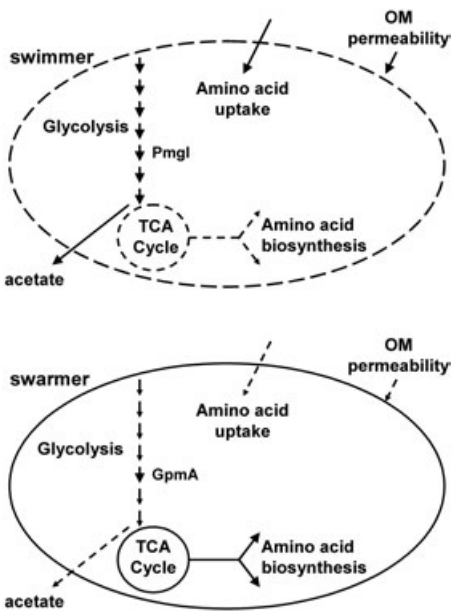

Fig. 5. Proposed model of the metabolic differentiation. When cells are grown in a nutrient-rich medium, there is an obvious advantage associated with importing biological compounds rather than expending excess energy for *de novo* biosynthesis. As reflected by the systemic changes in the metabolic circuit after differentiation, the swarmers do not follow this general metabolic trend [downregulated pathways (dotted arrows), upregulated pathways (bold arrows)]. These global changes may override the pairwise environmental input–responses that are typically observed under nutrient-controlled settings in an aqueous environment. Unlike the required conditions for initiating differentiation, nutritional requirements other than glucose are minimal to maintain the differentiated state. The pre-differentiated cells probably utilize the metabolic pathways of the swimmers, because the pH is significantly reduced in the centre of the swarm plate where the swarmer differentiation occurs (Toguchi *et al.*, 2000). OM, outer membrane.

In view of the classic paradigms of microbial growth established in aqueous environments, such metabolic behaviour may seem paradoxical, and some inferences made here should be tested directly by flux analysis. In

view of bacterial proliferation and persistence in the host, reduced uptake of exogenous nutrients and outer membrane permeability may represent a sophisticated survival strategy, which is also reflected in elevated resistance to the cationic peptides and a broad spectrum of antibiotics (Kim *et al*., 2003; Kim and Surette, 2003). The potential trade-off between reduced utilization of exogenous nutrients and resisting the host's innate anti-microbial mechanisms may confer an ecological advantage to *Salmonella*. Even with reduced uptake of exogenous nutrients, the systemic shift in metabolism maintains the growth rate of the swarmers comparable to that of the exponentially growing swimmers in aqueous environments. Thus, swarmer differentiation may represent a general adaptive response stimulated by the host environment. It should be noted, however, that not all swarming bacteria undergo a similar metabolic differentiation. Swarming cells of *Proteus* spp. also exhibit reduced uptake of exogenous nutrients and widespread changes in the patterns of enzyme activities and outer membrane protein composition; however, metabolic energy is derived from fermentation rather than respiration, which is primarily invested into flagellar activity rather than growth (Armitage, 1981; Falkinham & Hoffman, 1984).

It is well established that certain environmental stimuli can trigger bacteria to undergo differentiation in multicellular communities, such as fruiting bodies (Branda *et al*., 2001; Velice and Stredwick, 2002) and biofilms (Donlan, 2002; Sauer, 2003). A swarming population of *Salmonella* represents a unique example, and the metabolic differentiation described here illustrates that environmental stimuli can trigger a physiological differentiation to bring upon a global metabolic response, with the potential to override some of the well-established specific input–response relationships. Reassessment of the current paradigms in alternative models of bacterial growth outside the flask may yield additional dynamic and robust views on microbial pathogenesis and physiology.

Experimental procedures

Whole-cell protein preparation for 2DGE

One microlitre of an overnight culture of *S. enterica* serovar Typhimurium 14028 (ATCC 14028) was inoculated into the centre of the swim or swarm plates, composed of NBG (NB supplemented with 0.5% glucose) and 0 or 0.5% agar, respectively, and incubated at 37°C as previously described (Kim and Surette, 2003). When the swarm-front reached near the edges of the plate (≈9 h), the cells were suspended in 3 ml of fresh NBG by gently tilting the plates back and forth and harvested by centrifugation. With this method, the migrating cells easily lifted off the surface, whereas the vast majority of the cells in the centre of the plates remained intact on the surface. Exponentially growing swimmer cells were harvested at an OD_{600} of 0.25, resuspended in fresh NBG and spun down to similar pellet sizes as the swarmer samples. Each sample was lysed and nuclease-treated in sample buffers I and II, and solubilized in the urea/thiourea rehydration buffer (RB) as described elsewhere (Molloy *et al*., 2003), except the Biolytes were excluded and 2 mM tributylphosphine (TBP) replaced 50 mM DTT as the reducing reagent in RB. Protein concentration was estimated with the 2-D Quant Kit (Amersham Biosciences), and the final concentration of all samples were adjusted to 200 μg in 300 μl RB. The final concentrations of TBP and IPG buffer (Amersham Biosciences) were adjusted to 5 mM and 0.5% (v/v), respectively, and the samples were incubated at room temperature for 1 h. Subsequently, the samples were alkylated with 50 mM acrylamide for 2 h at room temperature.

2DGE and protein identification

All 2DGE-related equipment and reagents were obtained from Amersham Biosciences. Immobiline DryStrips (pH 3–11NL or pH 4–7) were passively rehydrated with the protein samples overnight at room temperature. The Ettan IPGphor was used for isoelectric focusing and 12% SDS-PAGE was carried out on the DALT*six* as described elsewhere (Molloy *et al*., 2003), except the second equilibration buffer contained 200 mM acrylamide instead of 3% iodoacetamide. The gels were washed twice each in double-distilled water and fix (10% ethanol, 7% acetic acid), before staining with SYPRO Ruby (Molecular Probes), colloidal Coomassie (GelCode Blue from Pierce) or glutaraldehyde-free silver (PlusOne from Amersham Biosciences), as recommended by the manufacturers. Each protein spot was quantified with the imaging software ImageJ (National Institutes of Health, USA). The range of variations in the quantitative differences were largely due to differences in the sensitivities of the stains used (i.e. silver, colloidal Coomassie, fluorescence), and less due to different samples of whole-cell extracts. The majority of protein spots that exhibited higher range of variations were from less abundant proteins (e.g. YfiD and AsnA), where the silver stain yielded the maximum fold-differences whereas the colloidal Coomassie-stained images revealed the minimum fold-differences. Spots of interest were digested with trypsin and their peptide mass fingerprint data were generated by the Voyager DE-STR MALDI-TOF mass spectrometer (PerSeptive Biosystems) as described elsewhere (Simpson, 2003). The online ProteinProspector (http://prospector.ucsf.edu) software was used to probe the non-redundant, all species database at NCBI, with a maximum allowed error of 30 p.p.m. Although the vast majority of the top potential matches was a protein from *Salmonella*, only those that surpassed the criteria of having a minimum of five peptide matches and a MOWSE score of greater than 10^3 were considered to be legitimate. The predicted pI and the Mw of the potential matches were also cross-referenced to the original 2DGE data.

Comparison of growth rates and intracellular levels of ATP

The growth rates of the two cell populations could not be accurately compared by the conventional methods of measuring optical density or plating out serial dilutions, because

the relative size of the cells vary and a single swarmer cell can represent multiple units of vegetative swimmer cells (Fraser and Hughes, 1999). To overcome these constraints, total DNA concentration was measured with the green fluorescence DNA stain Syto-9 (Molecular Probes). As a control, serial dilutions of swimmer cells were either plated out on solid LB or stained with Syto-9. Before staining, the cells were resuspended in 150 μl of sterile water, boiled for 4 min and cooled on ice. Each lysate was serially diluted 10-fold five times in 90 μl volumes in a 96-well clear-bottom black plates (Nunc) and incubated with 10 μl of 34 μM Syto-9 for 30 min. Fluorescence was measured in the Victor^2 1420 multilabel counter (Wallac) set at 0.1 s exposure with the fluorescein filter. For each sample, the fluorescence values from the five dilution series were plotted to extract the linear quantitative value. The relative differences in the fluorescence of the samples were found to accurately correspond with the cell number, validating this approach for monitoring growth. To measure changes in growth, multiple cultures of swimmers and swarmers were initiated, and the cells were harvested from the entire culture volume (swimmers) or the entire plate (swarmers) in duplicate, and processed as described above at each time interval.

Relative differences in the intracellular ATP levels of the two cell populations were compared with the ATP bioluminescence assay kit HS II (Roche) as per manufacturer's instructions. The kit estimates intracellular ATP levels as a function of the activity of the ATP-dependent luciferase when added to whole-cell lysates, and luminescence was measured in black-bottom 96-well plates (Nunc) with the Victor^2. Swimmer and swarmer cells were harvested in duplicate at four different time points during exponential growth as described above, and the luminescence values were normalized to the respective DNA concentrations. The normalized values were averaged to estimate the relative differences in the intracellular levels of ATP between the two cell populations. All experiments were repeated three times to ensure reproducibility of the results.

Transcriptional analyses of fumA *and* pykF *genes*

Luciferase-based transcriptional fusions of *fumA* and *pykF* genes were previously characterized as part of a random promoter library in serovar Typhimurium 14028 (Goh *et al*., 2002; Bjarnason *et al*., 2003). NBG swarm media were prepared as described above. Overnight cultures of serovar Typhimurium 14028 strains harbouring each transcriptional fusion were diluted in fresh NBG broth and 2×10^5 cfu in 200 μl volumes were gently spread-plated onto the swarm media, and the excess liquid was allowed to absorb into the media for 10 min at room temperature. Plates were incubated at 37°C, and the spread-plated cultures were harvested with 2 ml of fresh NBG at 4, 6 and 8 h for luminescence and DNA measurements. Luminescence was measured as previously described (Kim and Surette, 2003) and the DNA concentration of each sample was measured as described above, which was used to normalize the luminescence values.

To analyse spatial patterns of gene expression in actively migrating swarmers, the centres of swarm plates were inoculated and incubated as described for the proteomic studies. The swarm-front nearly reached the edges of the plate 9 h after centre inoculation (as opposed to spread-plating), and cells were harvested from the outer edges of the swarm-front or ≈1 cm outside the site of initial migration (i.e. inner edge) with sterile toothpicks into 200 μl of fresh NBG. Luminescence and DNA concentrations of the actively migrating swarmers were measured as described above, and all experiments were repeated at least twice.

Swarm assay and preparation of swarm plates containing hybrid media

Semi-solid plates (0.5% agar) were prepared with LB, LBG (LB with 0.5% glucose), M9G (M9 with 0.5% glucose), M9GC (M9G with 0.1% casamino acids), NB or NBG, and allowed to dry as described elsewhere (Kim and Surette, 2003). These plates were inoculated as described above, and incubated overnight at 37°C. We also attempted to induce swarming in modified M9G with reduced buffering power by varying phosphate levels. However, we did not detect any swarming even after 48 h (data not shown).

For the preparation of hybrid swarm plates, petroleum jelly was applied as a sealant to the rims of small Petri dishes (60 × 15 mm; Nunc) with a sterile cotton swab and placed inverted within the centre of large Petri dishes (150 × 15 mm; VWR). Fifty-five millilitres of semi-solid LB, LBG or M9G were poured around the inverted dish. Once the media solidified, the small dish was removed and the resulting space was filled with the NBG swarm medium (13 ml). The hybrid swarm plates were allowed dry overnight and the centre of the NBG portion was inoculated with an overnight culture and incubated as previously described (Kim and Surette, 2003). The swarm patterns were recorded 24 h later. The observed swarming of *Salmonella* on the NBG-M9G hybrid plates does not simply result from diffusion of nutrients from the NBG into the M9G. If this were the case, we would expect glucose diffusion from NBG to LB and swarming on those plates and this is not observed. Moreover, diffusion would not be expected to result in distribution of nutrients across the plate in the time frame of this experiment (less than 24 h).

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