Beyond Antimicrobial Resistance: Evidence for a Distinct Role of the AcrD Efflux Pump in Salmonella Biology

Michelle M. C. Buckner,* Jessica M. A. Blair,* Roberto M. La Ragione,† Jane Newcombe,‡ Daniel J. Dwyer,§ Alasdair Ivens,* Laura J. V. Piddock*

Antimicrobials Research Group, Institute of Microbiology and Infection, College of Medical and Dental Sciences, The University of Birmingham, Edgbaston, Birmingham, United Kingdom; School of Veterinary Medicine, Faculty of Health and Medical Sciences, University of Surrey, Guildford, Surrey, United Kingdom; School of Biosciences and Medicine, Faculty of Health and Medical Sciences, University of Surrey, Guildford, Surrey, United Kingdom; Department of Cell Biology and Molecular Genetics, Institute for Physical Science and Technology, Department of Bioengineering, Maryland Pathogen Research Institute, University of Maryland, College Park, Maryland, USA; Centre for Immunity, Infection and Evolution, University of Edinburgh, Edinburgh, Scotland

ABSTRACT For over 20 years, bacterial multidrug resistance (MDR) efflux pumps have been studied because of their impact on resistance to antimicrobials. However, critical questions remain, including why produce efflux pumps under non-antimicrobial treatment conditions, and why have multiple pumps if their only purpose is antimicrobial efflux? Salmonella spp. has five efflux pump families, including the resistance-nodulation-division (RND) efflux pumps. Notably, the RND efflux pump AcrD has a unique substrate profile, distinct from other Salmonella efflux pumps. Here we show that inactivation of acrD results in a profoundly altered transcriptome and modulation of pathways integral to Salmonella biology. The most significant transcriptome changes were central metabolism related, with additional changes observed in pathogenicity, environmental sensing, and stress response pathway expression. The extent of tricarboxylic acid cycle and fumarate metabolism expression changes led us to hypothesize that acrD inactivation may result in motility defects due to perturbation of metabolite concentrations, such as fumarate, for which a role in motility has been established. Despite minimal detectable changes in flagellar gene expression, we found that an acrD mutant Salmonella enterica serovar Typhimurium isolate was significantly impaired for swarming motility, which was restored by addition of fumarate. The acrD mutant outcompeted the wild type in fitness experiments. The results of these diverse experiments provide strong evidence that the AcrD efflux pump is not simply a redundant system providing resistance resilience, but also has distinct physiological functions. Together, these data indicate that the AcrD efflux pump has a significant and previously underappreciated impact on bacterial biology, despite only minor perturbations of antibiotic resistance profiles.

IMPORTANCE Efflux pumps in Gram-negative bacteria are studied because of their important contributions to antimicrobial resistance. However, the role of these pumps in bacterial biology has remained surprisingly elusive. Here, we provide evidence that loss of the AcrD efflux pump significantly impacts the physiology of Salmonella enterica serovar Typhimurium. Inactivation of acrD led to changes in the expression of 403 genes involved in fundamental processes, including basic metabolism, virulence, and stress responses. Pathways such as these allow Salmonella to grow, survive in the environment, and cause disease. Indeed, our data show that the acrD mutant is more fit than wild-type Salmonella under standard lab conditions. We hypothesized that inactivation of acrD would alter levels of bacterial metabolites, impacting traits such as swarming motility. We demonstrated this by exogenous addition of the metabolite fumarate, which partially restored the acrD mutant’s swarming defect. This work extends our understanding of the role of bacterial efflux pumps.

Received 14 October 2016 Accepted 21 October 2016 Published 22 November 2016
Citation Buckner MMC, Blair JMA, La Ragione RM, Newcombe J, Dwyer DJ, Ivens A, Piddock LJV. 2016. Beyond antimicrobial resistance: evidence for a distinct role of the AcrD efflux pump in Salmonella biology. mBio 7(6):e01916-16. doi:10.1128/mBio.01916-16.
Editor Julian E. Davies, University of British Columbia
Copyright © 2016 Buckner et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.
Address correspondence to Laura J. V. Piddock, l.j.v.piddock@bham.ac.uk.

Resistance-nodulation-division (RND) efflux pumps in Gram-negative bacteria confer intrinsic multidrug resistance (MDR) by exporting a broad range of antimicrobial compounds out of the bacterial cell. Traditionally, Enterobacteriaceae such as Escherichia coli and Salmonella have five families of efflux pumps, the ABC and MFS superfamilies and the SMR, MATE, and RND families (1). Recently, the PACE family of efflux pumps was discovered in Acinetobacter species and is present in a range of Proteobacteria (2, 3). Salmonella enterica serovar Typhimurium (here, Salmonella Typhimurium) is used extensively as a model pathogen for many reasons, including easy genetic manipulation, good infection models, and relevance to other pathogens (4, 5). Salmonella Typhimurium has five RND MDR efflux systems: AcrAB, AcrAD, AcrEF, MdtABC, and MdsABC (5, 6). AcrB, and its homologues in other Gram-negative pathogens (e.g., MexB in Pseudomonas aeruginosa and CmeB in Campylobacter jejuni) is considered the most important RND system to human health because it transports a wide range of structurally varied antimicrobials and is...
more abundant within the cell than other efflux pumps (for a
review, see reference 7). Furthermore, inactivation of acrB in Sal-
monella (and its homologues in other bacteria) confers multidrug
hypersusceptibility, while single deletions of the other RND efflux
pump genes have little or no effect on susceptibility to most anti-
microbial agents (5, 8, 9). Interestingly, AcrD has 70% nucleotide
and 79% amino acid similarity with AcrB (9), yet it possesses a
distinct substrate profile that includes aminoglycoside antibiotics
(10, 11). AcrB and AcrD also differ in the structures of their prox-
imal binding pockets, which may underpin the differences in their
substrate profiles (12). We have previously shown that the AcrD
efflux pump impacts biofilm formation, as evidenced by an acrD
mutant that showed significantly reduced biofilm formation and
reduced expression of key biofilm proteins encoded by csgBD (13).

Little is known about the natural and potentially homeostatic
functions of efflux pumps in pathogen biology. Efflux pumps are
 evolutionarily ancient proteins which long predate the use of an-
tibiotics (14). In Salmonella, the AcrAB-TolC efflux pump is in-
volved in protection from and efflux of bile and toxic bile salts
(15–21). In E. coli, a physiological substrate is the siderophore
enterobactin, which is effluxed by AcrAB-TolC, AcrAD-TolC, and
MdtABC pumps (22–24). To further understand the natural func-
tion of AcrB in Salmonella, we previously determined the tran-
scriptome of a Salmonella acrB mutant; the effect was profound and
 correlated with an altered basic biology of the organism (25).
Furthermore, we previously showed that inactivation of individ-
ual or multiple acr efflux pump genes results in increased expres-
sion of the remaining efflux pump genes, suggesting a sensing
mechanism to detect and regulate relative expression levels of the
different pumps (9). When acrB was deleted, acrD expression in-
creased (9). While the physiological role of AcrB is becoming
clearer, much less is known about the functions of AcrD. There-
fore, we sought to understand how loss of AcrD impacted the
biology of the organism and whether loss of this pump led to
changes to Salmonella physiology.

In the current study, we determined the transcriptome of a
Salmonella mutant, constructed and tested previously (8, 9), in
which acrD had been genetically inactivated. Here, we demon-
strate that multiple genes, including those involved in metabo-
lism, stress responses, and virulence, were altered by acrD inacti-
vation. Using these transcriptomic data as a guide, we examined
phenotypic differences between the parental strain and the acrD
mutant. Specifically, we explored the ability of an acrD Salmonella
mutant to swim and swarm, grow on various carbon sources, grow
under anaerobic conditions, and invade polarized epithelial cells,
and we tested the fitness of the acrD mutant relative to the parental
strain. Our data suggest that AcrD has a different role in the biol-
ogy of the organism than previously assumed.

RESULTS

Inactivation of acrD leads to distinct transcriptional changes.
The transcriptome of the acrD mutant revealed significantly al-
tered expression of 403 genes compared to the parental SL1344
strain (for a list of genes with significantly altered expression, see
Table S1 in the supplemental material; full microarray data are
available at http://www.ebi.ac.uk/arrayexpress/experiments/E-
MEXP-2975/samples/). Specifically, in the acrD mutant expres-
sion of genes encoding proteins involved in anaerobic growth,
ATP synthesis, amino acid metabolism, sugar transport, glycol-
sis/gluconeogenesis, ribosomal subunit biosynthesis, RNA poly-
merase, and oxidative phosphorylation were generally increased
(Fig. 1). Expression of genes associated with pathogenicity, sigma
factors, stress response, the tricarboxylic acid (TCA) cycle, and
purine metabolism were generally decreased (Fig. 1). Surprisingly,
very few of the transcriptomic changes could be directly linked to
drug export.

It seemed unlikely that the changes observed at the transcrip-
tomic level were simply due to the lack of a large membrane pro-
ten. If this were the case, we would expect the deletion of acrD and
acrB to result in similar changes and also changes in membrane
stress response genes, e.g., cpxAR and baeSR (26–28). Both CpxR and BaeR are known to induce expression of efflux pumps, including AcrD (26, 29, 30). In E. coli, inactivation of tolC activates the Cpx and Bae pathways (28). However, there was no significant change in expression of either the cpx or bae gene in the acrD mutant.

**Carbon metabolism is largely unaltered by loss of acrD.** Since many of the observed transcriptional changes involved genes that encode products involved in carbon utilization, the Biolog phenotypic microarray system (Biolog, Inc., USA) was used to measure the respiration of the wild type and acrD mutant on various carbon sources (PM1 and PM2A). The respiration levels on different carbon sources for the acrD mutant and the wild-type strain were very similar, with only one major difference observed. The acrD mutant had significantly higher respiration levels on saccharate relative to that of wild-type Salmonella ($P = 0.0037$).

In order to validate the results from the phenotypic microarrays and to determine if the change in respiration was also associated with a change in growth, selected carbon sources were tested. Growth in M9 minimal medium supplemented with glucose, fructose, succinate, aspartate, pyruvate, or saccharate was examined (see Fig. S1 in the supplemental material). As a control, growth of the acrD mutant and wild-type Salmonella in LB broth was measured, and no differences were found in generation time or the final optical density (OD) at 19 h (Fig. S2). The generation time of the acrD mutant was not significantly different when grown with glucose, fructose, succinate, aspartate, pyruvate, or saccharate as a carbon source (Fig. S3a). Likewise, measurement of the final OD of the culture indicated that the acrD mutant and wild-type strain grew to similar final densities on the carbon sources tested (Fig. S3b).

**Inactivation of acrD leads to increased expression of nitrate reductase and nitrite reductase genes.** In the acrD mutant, the expression of six genes (napACF and nirBCD) involved in anaerobic growth was increased between 3.06- and 22.83-fold (Table S1). However, under anaerobic conditions, there was no significant difference in the generation time (Fig. S4a) or the final OD (Fig. S4b) of the acrD mutant culture compared to the wild-type parental strain. Additionally, there was no difference in growth in 1 mM sodium nitrate, nor was there a difference in survival in 10 mM acidified sodium nitrite (data not shown).

**Inactivation of acrD results in reduced virulence gene expression.** Inactivation of acrD led to decreased expression of 13 genes known to be involved in Salmonella virulence. The expression levels of genes from Salmonella pathogenicity islands 1 (SPI-1), -2, -3, -10, and -18 were significantly decreased (0.05- to 0.5-fold, which corresponds to a 20- to 2-fold reduction) (see Table S1). This included reduced expression of genes encoding secreted proteins (cigR and sseBCDE) and virulence protein chaperones (sicP and sscAB), while expression of a gene encoding a needle apparatus sorting platform component (orgA) was increased.

Genes from SPI-1 have been shown to be expressed by only a portion of the total population (31–33), so techniques such as microarrays and reverse transcription-PCR (RT-PCR), which measure gene expression across a whole population, only provide partial information about gene expression of individual bacteria in a population. The expression of the SPI-1 gene prgH, which encodes the essential basal component of the needle complex of the SPI-1 type III secretion system, has been used previously to measure expression of SPI-1 in single cells (31, 34). Therefore, strains containing the promoter of prgH (SPI-1) fused to gfp were analyzed by flow cytometry and the number of fluorescent and nonfluorescent cells were enumerated. (a) Mean results ± standard deviations. *, $P < 0.05$. (b) A representative example of the flow cytometry results for SL1344 and SL1344 acrD::aph cells.
correlate with the decreased detection of SPI-1 transcripts in the acrD mutant microarray analysis (Table S1).

Intriguingly, this reduction in the proportion of bacteria expressing SPI-1 did not correlate with decreased invasion of the acrD mutant into Caco-2 polarized epithelial cells. There was no difference in the virulence of the acrD mutant compared to the parental SL1344 strain; as there was no difference between wild-type SL1344 and the acrD::aph strain in association (2 h), invasion (4 h), and persistence (8 and 24 h) in Caco-2 polarized epithelial cells ($P < 0.05$, $P = 0.745$, $P = 0.846$, $P = 0.942$, and $P = 0.685$, respectively) (Fig. S5).

Inactivation of acrD reduces motility. Compared to SL1344, the acrD mutant was impaired for swimming. SL1344 swam to cover an average area of 471.5 mm$^2$, while the acrD mutant covered 266.7 mm$^2$ ($P = 0.0018$) (Fig. 3a). Likewise, the acrD mutant was impaired for swarming compared with SL1344, which covered an average area of 6,090 mm$^2$ while the acrD mutant covered 510 mm$^2$ ($P < 0.001$) (Fig. 3b). The swimming assay produces results that are inherently variable, and so we minimized this impact by performing 13 replicates.

Since there were no changes in expression of common motility genes found in the transcriptome (e.g., flagellar genes), yet we observed a drastic impact on motility, we explored the effect of metabolites on motility. This was because expression levels of many metabolism-associated genes were altered in the transcriptome. Fumarate has been shown to impact the switching of the direction of flagellum rotation (35–37). Furthermore, the transcriptomic data showed increased expression of frdABCD and aspA, as well as decreased expression of sdhCDA and fumC (see Table S1), the gene products of which impact fumarate levels. To determine if fumarate could restore the motility defect seen in the acrD mutant, exogenous fumarate was added to the culture medium. At concentrations of 0.1, 0.25, 0.5, 1, 2, and 4 mM, fumarate inhibited swimming motility of wild-type SL1344 ($P = 0.004$, $P <
0.001, \( P < 0.001, P < 0.001, P < 0.001, P = 0.0016 \), respectively), while concentrations of 0.01 and lower did not impact swimming (Fig. 3a). For the \( acrD \) mutant, concentrations of 0.25, 0.5, 1, 2, and 4 mM fumarate inhibited swimming motility (\( P = 0.015, P = 0.041, P = 0.016, P = 0.020, P = 0.018 \), respectively) (Fig. 3a). Fumarate concentrations between 0.1 and 0.5 mM increased the swarming ability of the \( acrD \) mutant (\( P < 0.05 \) for 0.1, 0.25, and 0.5 mM fumarate) (Fig. 3b) compared with LB alone. Our data indicate that the ability to swarm is either on or off. In LB alone, 9 replicates showed that the wild type swarmed very well and only one replicate showed moderate swarming; for the \( acrD \) mutant, 8 replicates did not swarm at all and 2 replicates swarmed moderately (Fig. 3b). At concentrations of 0.1, 0.25, and 0.5 mM fumarate, the proportion of \( acrD \) mutants which did swarm increased \( (P < 0.05) \) (Fig. 3b). At 4 mM fumarate, swarming of the wild type was inhibited \( (P < 0.001) \) (Fig. 3b). One limitation of this experiment was that the swarming wild-type bacteria reached the edge of the plate, and we were unable to determine if fumarate increased swarming of SL1344 by the same magnitude as in the mutant.

**Inactivation of \( acrD \) impacts bacterial fitness.** Since no differences were seen in growth when evaluated under a variety of conditions, yet expression levels of 403 genes were altered, we hypothesized that these numerous gene expression changes would alter the fitness of the \( acrD \) mutant in a competitive environment. Bacterial fitness can be determined in a number of different ways; initially we tested fitness under standard laboratory conditions *in vitro* (LB broth, 37°C with aeration, inoculation with a 50:50 ratio of wild-type *Salmonella* to \( acrD \) mutant). Under these conditions, the \( acrD \) mutant outcompeted the wild type as early as 4 h postinoculation (Fig. 4a). After 2 h of growth, the \( acrD \) mutant composed 57% of the population, and by 4 h 92% of the bacteria were \( acrD \) mutants \( (P < 0.001) \). This high proportion rose to 97% at 6 h \( (P < 0.001) \) and then varied between 96% and 99% \( (P < 0.001) \) for the remainder of the 72 h, which included passaging into fresh medium at 12, 24, and 48 h.

To measure fitness under *in vivo* conditions, competitive infection experiments were carried out. Polarized Caco-2 epithelial cells were infected with a 50:50 ratio of wild-type *Salmonella* to the \( acrD \) mutant. At 2, 4, 8, and 24 h postinfection, the proportion of the \( acrD \) mutant to the wild-type SL1344 was not significantly altered (Fig. 4b). This indicated there was no fitness differential associated with the loss of \( acrD \) during the infection of polarized Caco-2 epithelial cells (Fig. 4b).

**DISCUSSION**

Our data suggest that the AcrD efflux pump has a unique biological role. This was demonstrated by the significant changes observed in the transcriptome. Comparison of the transcriptomes of the \( acrD \) mutant (presented in this work; further information is available at [http://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-2975/samples/](http://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-2975/samples/)) with the previously published \( acrB \) mutant transcriptome (25) showed that the effect was very distinct, which supports the hypothesis that AcrD is not a “backup” efflux pump but has its own physiological purpose in the cell. This comparison identified 232 significant gene expression changes specific to the inactivation of \( acrD \) and that were not affected by the disruption of \( acrB \). Compared to the \( acrB \) mutant transcriptome, 169 genes were differentially expressed by both \( acrB \) and \( acrD \) mutants. Of these, expression of 91 genes was altered in the same way (e.g., increased in both mutants), and 78 genes were expressed in an opposite manner (e.g., increased in one but decreased in the other). There is experimental evidence that AcrB and AcrD efflux pumps have distinctive substrate profiles with respect to aminoglycoside antibiotics (10–12). Our data showed that deletion of either \( acrB \) or \( acrD \) has different impacts on the bacterial transcriptome, which supports the suggestion that these pumps have distinct roles. We speculate that accumulation of certain metabolic intermediates in the bacterial cell may trigger feedback mechanisms which alter gene expression and metabolism in different ways (23, 38).

Some of the genes identified in the microarray were drastically altered in the \( acrD \) mutant; for example, *adh* expression increased...
70-fold and fruB increased 45-fold. On the other hand, some genes had <10-fold changes but these changes were still statistically significant. These data are included in Table S1 in the supplemental material for several reasons. First, depending on gene function, small changes in expression can translate into significant changes in the biology of the organism (39, 40). Second, multiple small changes which occur in the same or related pathways can have strong impacts on phenotypes (41, 42). In the data presented here, the expression changes in genes involved in fumarate metabolism (frdBACD and sdhCDA) were below 10-fold, yet fumarate had a direct and significant impact on the acrD mutant’s motility (Fig. 3). Third, these data, including the large and small changes, will likely prove useful for the scientific community.

Nearly 100 genes associated with metabolism were altered by the inactivation of acrD. Table S1 lists genes with significantly altered expression; selected genes and operons are discussed below. Expression of the pyruvate formate lyase I gene, pfIB, increased. PfIB is involved in glucose metabolism, converting pyruvate to formate and acetate, and is preferentially used under anaerobic conditions (43, 44). Abernathy et al. (44) found that deletion of pfIB led to increased intracellular replication in intestinal epithelial cells as a result of increased SPI-1 expression. In our study, the acrD mutant had increased pfIB expression and reduced SPI-1 expression, indicating another connection between efflux pump gene expression, metabolism, and virulence. The fructose operon (fruBKA), under the control of FruR (45), was upregulated in the acrD mutant. However, there was no difference between growth of the acrD mutant and wild type when fructose was used as a carbon source. Another metabolism gene, ilvC, had higher expression in the acrD mutant. IlvC is a component of the pathway Salmonella uses to synthesize isoleucine and valine, and it is encoded by ilvGEDAYC (46). IlvGEDA is an operon, but transcription of ilvC is separate and dependent on IlvY (46). We were surprised that the Biolog phenotypic microarray data did not indicate significantly altered respiration of the acrD strain on carbon sources other than saccharate. We hypothesize that the effect of the transcriptional changes may be compensatory changes within the mutant to ameliorate the effect of loss of AcrD.

Six genes, napACF and nirBCD, associated with nitrate and nitrite reduction were upregulated when acrD was inactivated. This contrasts with the reduction in expression of these same genes in the acrB mutant (25). Salmonella has two nitrate reductases, one located in the cytoplasm and the other in the periplasm (NapACF) (47). NirBD is an NADH-dependent nitrite reductase located in the cytoplasm, while NirC is a nitrite/proton antiporter in the bacterial membrane, that allows transport of nitrite into the cytoplasm for detoxification (48, 49), and contributes to Salmonella virulence in both mice and macrophages (50). However, there was no difference in the ability of the acrD mutant to grow anaerobically compared to wild-type Salmonella, nor was there a significant difference in the ability of the mutant to grow in the presence of sodium nitrate or survive in acidified nitrite. It seems plausible that deletion of these genes, as in studies performed by other groups, has a greater effect on phenotype than the level of the increased expression (3.5- to 23-fold) seen in our acrD mutant.

Among the differences in the transcriptomes, the expression levels of genes involved in virulence were reduced in the acrD mutant. Salmonella has several pathogenicity islands, including SPI-1 and SPI-2, which contribute to invasion of nonphagocytic cells as well as survival and replication within host cells (4, 51). The SPI-1-associated genes, which were downregulated in the acrD mutant, were different from the SPI-1 genes downregulated in the acrB mutant (25). Our transcriptomic data indicate that inactivation of acrD led to reduced expression of SPI-1- and SPI-2-associated chaperones, translocon components, and effectors. Despite the reduced expression of some SPI-1-associated genes and the reduced proportion of bacteria expressing the SPI-1 apparatus, neither association, invasion, nor persistence in Caco-2 treated cells was impacted by the loss of acrD. A previous study also showed no impact of acrD inactivation on adhesion or invasion of INT-407 epithelial cells or on colonization and persistence of 1-day-old and 2-week-old chicks between the acrD mutant and wild-type strain SL1344 (52). In line with these findings, Nishino et al. (2006) showed inactivation of acrD had minimal impact on BALB/c mouse survival (5). In contradiction to the findings of Buckley et al. (52) and Nishino et al. in 2006 (5), another study found that the acrD strain was attenuated in an INT-407 model of infection (9). However the polarized Caco-2 epithelial cells used in our study are more physiologically relevant. Furthermore, our competitive infection data confirm that the acrD mutant is as fit as wild-type Salmonella during infection. Salmonella invasion of polarized epithelial cells occurs in a highly cooperative manner with multiple bacteria engulfed by Salmonella induced membrane ruffles (53). We hypothesize that this cooperative entry accounts for this apparent contradiction, as cells that express SPI-1 induce ruffles, which engulf both SPI-1 expressing and non-SPI-1-expressing bacteria.

The acrD mutant was able to outcompete the wild-type Salmonella in vitro in the fitness experiment in LB culture medium. We postulate that the metabolic shifts highlighted by the transcriptomic data provide an advantage to the acrD mutant in the LB fitness experiment. The cumulative effect of changes to metabolic gene expression does not impair the ability of the mutant to outcompete the parental strain.

Salmonella species are highly motile, and are capable of swimming and swarming in a flagella dependent manner (54, 55). Swimming is associated with counterclockwise rotation of the flagella, and is often interspersed with tumbles, brought about by switching direction of flagellar rotation (56). This ability to switch rotation direction is also important for swarming motility, and is controlled by the chemotaxis response regulator CheY (56). Swarming Salmonella move within a wet “slime” layer composed of polysaccharides, surfactants, and peptides (57, 58). In a gene expression study conducted by Wang et al. 2004 (57), the flagella operons were not upregulated during swarming. Swarming Salmonella also have distinct changes in basic metabolism, with glucose a key energy source for the process (59). We made similar observations in this study; expression of multiple metabolic but not flagella specific genes was altered in the acrD mutant, yet significant changes to swarming were detected.

Recent studies have focused on finding novel genes and/or pathways which mediate swimming (60-62). A screen of a mutant library containing 1023 mutants in S. typhimurium 14028s found 21 mutants with impaired swimming and swarming, forty nine with impaired swimming, but normal swarming, and also 49 with impaired swimming but normal swarming, and 39 hyper-motile mutants (61). Further exploration of these novel motility genes identified by Bogomolnaya et al. 2014 is needed. Future work un-
covering more pathways and proteins directly involved in swarming may shed further light on our data.

Despite the drastic reduction in swarming motility of the acrD mutant, we did not see significant changes in the expression of genes known to impact motility and swarming, including cheY. We observed significant changes in expression of genes associated with the production of fumarate. The key enzymes in the interconversion of succinate and fumarate are FrdABCD and SdhCDAB (63). Under aerobic conditions, sdh is upregulated and these proteins catalyze the oxidation of succinate at higher frequency (64). In the acrD mutant, the sdhCDAB genes were downregulated. Under anaerobic conditions, frd genes are upregulated and fumarate can act as a terminal electron acceptor; however it is a low energy yielding acceptor (63–66). In the acrD mutant the frdABCD operon was upregulated, yet no difference in anaerobic growth was seen. Together with the upregulation of aspA and downregulation of fumC we hypothesized that the acrD mutant has altered intracellular levels of fumarate.

Fumarate is associated with flagella rotation switching in a model which uses cytoplasm free envelopes of both Salmonella and E. coli where flagella are present, but do not normally change directions (35). However, the addition of 1 mM fumarate restores the switching of flagella, even in the absence of CheY (35, 37). With the addition of fumarate the flagella rotated in the clock-wise direction more often; fumarate was shown to target the switch motor complex (37). In line with these findings, when we added fumarate (0.001 to 4 mM) to the swarming assay medium, there was a clear increase in swarming of the acrD mutant at concentrations of 0.1 to 0.5 mM. The intracellular concentration of fumarate in S. typhimurium has previously been shown to be 0.23 μM (± 0.12) (67), thus the exogenous levels of fumarate needed to impact swarming (0.1 to 1 mM) are higher than those found within the cell. Furthermore, there was an “all-or-nothing” swarming phenotype, and the addition of fumarate increased the proportion of the mutant bacteria that swarmed. Taken together, we hypothesize that inactivation of the AcrD efflux pump leads to altered levels of fumarate, which directly impacts swarming motility.

A recent elegant study examined the localization of AcrB, AcrD and TolC within the membrane (68). In this study they found that AcrB and AcrD formed stabilized foci when bound to TolC and that as levels of AcrD increased, AcrB was displaced from TolC, a phenomenon the authors call transporter exchange (68). This exchange did not occur when AcrB substrates were present (68). Extrapolating this hypothesis provides a potential explanation of our data; when acrD is deleted there are fewer competitors for AcrB to bind with TolC and therefore, the AcrAB-TolC complex is stabilized, even in the absence of AcrB substrates. We postulate this could affect the metabolite concentrations within the cell.

In summary, our study demonstrates important and significant differences in bacterial gene expression occur as a result of inactivation of acrD, and this response is different to the response elicited by the inactivation of acrB. This work highlights a previously underappreciated role of AcrD in the fundamental biology of Salmonella.

**MATERIALS AND METHODS**

**Bacterial strains and culture media.** All strains were derived from Salmonella enterica serovar Typhimurium SL1344 (69), and the efflux pump-inactivated mutant (SL1344 acrD::aph) has been previously described (8, 9). LB broth (Sigma-Aldrich, United Kingdom), M9 minimal medium (components from Sigma-Aldrich, United Kingdom), and morpholinopropanesulfonic acid (MOPS) minimal medium supplemented with 0.2% glucose (Teknova, USA) were used throughout this study.

**Microarrays.** Microarray experiments were carried out and results were analyzed exactly as described previously for other strains; they were carried out in parallel to those with an SL1344 acrB::aph mutant (25, 70). Briefly, overnight cultures of S. Typhimurium SL1344 and the mutant strain were grown in MOPS minimal medium (glucose) at 37°C until early logarithmic phase (OD600 ≈ 0.7). For each strain, three biological and two technical replicate RNA preparations were made. Data were analyzed with the Bioconductor (71) and Pathway Tools (72) programs. Data with a B (log odds value) value of ≥0, which corresponds to an adjusted P value of <0.004, were considered significant. The microarray data set is available at http://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-2975/samples/ (submitted 10 November 2010, released 1 June 2011, last updated 2 May 2014; minimal medium used [http://www.ebi.ac.uk/arrayexpress/protocols/87504/?ref=E-MEXP-2975]).

**Phenotypic microarrays.** Salmonella Typhimurium strains were cultured from frozen (~80°C) stocks on LB agar for 24 h at 37°C, aerobically. Bacteria were harvested from agar plates and resuspended into IF-0a inoculating fluid (Biolog, Inc., USA) and adjusted to 42% transmittance (turbidimeter; Biolog). A working cell suspension of 10 ml IF-0a, 2 ml of bacterial suspension, and 120 μl of tetrazolium dye (90 μl dye D, 30 μl dye F; Biolog) was made for the PM1 and PM2A carbon utilization plates. One hundred microliters of suspension was added to each of the 96 wells on the appropriate PM plates. The inoculated PM plates were placed in an Omnilog automatic plate reader (Biolog) and incubated at 37°C, aerobically, for 48 h. Metabolism of the various carbon sources was recorded every 15 min based on the reduction of the tetrazolium violet redox dye, which produces a purple color indicative of active bacterial respiration. Abiotic negative-control plates indicated false-positive results due to autocorrection of the dye observed in PM1 with l-arabinose, D-xyllose, D-ribose, or l-lyxose. False-positive results observed in PM2A included D-arabinose, 2-deoxy-D-ribose, D-glucosamine, 5-keto-D-gluconic acid, and dihydroxyacetone.

Each PM plate preparation was repeated in triplicate for each strain. The values of the experimental replicates for each strain for each carbon and nitrogen substrate were compared by a one-way analysis of variance and Tukey post hoc multiple-comparison test, using a 95% family-wise confidence level. Statistical significance was assigned at the P ≤ 0.05 level.

**Bacterial growth in different carbon sources.** Bacteria were grown aerobically at 37°C for approximately 16 h in LB broth and then diluted to ~10^6 CFU/ml in fresh M9 minimal medium. For each strain, three biological and two technical replicates of each strain and are presented as the average optical density over time. For succinate, pyruvate, and saccharate (0.4%, aspartate (0.4%), pyruvate (0.2%), and saccharate (0.05%) (Sigma-Aldrich, United Kingdom). These concentrations were chosen based on preliminary experiments which determined the concentrations at which bacteria were able to grow. For glucose, fructose, and aspartate, absorbance was measured at 600 nm in a FLUOstar Optima apparatus (BMG Labtech, United Kingdom) at 37°C with agitation before each read, which was taken every 5 min for 19 h. Data were collected from three biological and three technical replicates of each strain and are presented as the average optical density over time. For fructose, pyruvate, and saccharate, growth in the FLUOstar was poor, and therefore growth kinetics were determined by inoculating 10 ml M9 minimal medium with histidine and the specified carbon source. Cultures were incubated at 37°C with agitation, and absorbance at 600 nm (OD600) was measured initially and at 2, 4, 6, 8, 10, 12, and 24 h. Final density (24 h) and generation time during exponential phase were calculated and analyzed using a two-tailed Student’s t test. P values of ≤0.05 were considered significant.
**An aerobic growth.** Cultures were set up in an anaerobic chamber (anaerobic gas growth mixture of 10% CO\(_2\), 10% H\(_2\), 80% N\(_2\)) in MOPS minimal medium supplemented with glucose and 6 mM L-histidine and incubated at 37°C for 24 h. Growth curves in MOPS minimal medium with histidine were started with 4% inoculum from overnight cultures grown anaerobically, and the initial OD\(_{600}\) was measured. The OD\(_{600}\) was measured every 2 h for the first 10 h and again at 24 h. Three independent experiments with three biological replicates were completed. Data are presented as the mean generation time ± the standard deviation. P values of ≤0.05 from a two-tailed Student’s t test were considered significant.

**Motility assays.** Bacterial strains were grown overnight at 37°C in LB broth with or without antibiotic, as appropriate. LB broth was used be incubated at 37°C for 24 h. Growth curves in MOPS minimal medium presented as the mean generation time

\[ P = \frac{t}{P} \]

and Syngene Transilluminator box, and ImageJ software was used to quantify plates, which were incubated for 16 h at 37°C. Images were taken using a velvet squares, colonies were replica plated onto LB agar containing 50 μg/ml of the mutant

\[ \text{Kanamycin-resistant colonies were enumerated.} \]

Using velvet squares, colonies were replica plated onto LB agar containing 50 μg/ml kanamycin and then incubated for 16 h, aerobically. Kanamycin-resistant colonies were enumerated.

Data are presented as the mean CFU of three independent experiments with four replicates each, ± standard deviations and were analyzed using a two-tailed Student’s t test. P values of ≤0.05 were considered significant.

**Competition assays.** Bacterial strains were grown statically at 37°C overnight in LB broth with or without antibiotic, as appropriate. Cells were washed in PBS, and the cultures were adjusted until the OD\(_{600}\) was 0.3. Wild-type SL1344 (100 μl) and mutant acrD::aph cells were added to 10 ml of LB broth (no antibiotics) and incubated at 37°C with aeration. Fresh broth was inoculated every 24 h. CFU were determined by serial dilution and plating on LB agar plates at 0 h (inoculum) and 2, 4, 6, 8, 10, 12, 24, 48, and 72 h. Replica plating with velvet squares onto LB agar plates containing 50 μg/ml of kanamycin was performed to determine the ratio of wild-type to mutant cells, and competition indices were determined. Three independent experiments were completed, with three biological replicates each. Data are presented as mean CFU ± standard deviations and analyzed using a two-tailed Student’s t test. P values of ≤0.05 were considered significant.

**SPI-1 reporter assays.** The chromosomal gfp reporter fused to the promoter of prgH has been previously described (31), and we have previously used gfp with the prgH promoter to measure activity of SPI-1 in individual cells (34). In brief, strains SL1344 prgH::gfp and SL1344 acrB: apb prgH: gfp were grown to mid-logarithmic phase in MOPS minimal medium. The cells were harvested by centrifugation at 2,200 x g at room temperature, washed, and resuspended in PBS. Bacteria were analyzed by flow cytometry using a FACSAria2 system (BD Biosciences). Cells were illuminated with a 488-nm laser, and scatter and GFP fluorescence data were collected through a 502 LP mirror and 530/30 bandpass filter. For each sample, 10,000 events were collected.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01916-16/-/DCSupplemental.

Figure S1, EPS file, 1 MB.
Figure S2, EPS file, 0.6 MB.
Figure S3, EPS file, 0.9 MB.
Figure S4, EPS file, 0.6 MB.
Table S1, DOCX file, 0.02 MB.

**ACKNOWLEDGMENTS**

We thank Andrew Bailey for carrying out the microarray experiments and Mark Webber, Xuan Wang Kan, Lee Rosner, and William Shafer for reading and providing constructive criticism of earlier versions of this manuscript.

**FUNDING INFORMATION**

This work was funded in part by RCUK | Medical Research Council (MRC) (G0501415).

**REFERENCES**

1. Anes J, McCusker MP, Fanning S, Martins M. 2015. The ins and outs of RND efflux pumps in *Escherichia coli*. Front Microbiol 6:587. [http://dx.doi.org/10.3389/fmicb.2015.00587](http://dx.doi.org/10.3389/fmicb.2015.00587).

2. Hassan KA, Jackson SM, Penesyan A, Patching SG, Tetu SG, Eijkelkamp BA, Brown MH, Henderson PJ, Paulsen IT. 2013. Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins. Proc Natl Acad Sci U S A 110:20254–20259. [http://dx.doi.org/10.1073/pnas.1307521110](http://dx.doi.org/10.1073/pnas.1307521110).

3. Hassan KA, Liu Q, Henderson PJ, Paulsen IT. 2015. Homologs of the Acinetobacter baumannii Acel transporter represent a new family of bacterial multidrug efflux systems. mBio 6:e01982-14. [http://dx.doi.org/10.1128/mBio.01982-14](http://dx.doi.org/10.1128/mBio.01982-14).

4. Buckner MM, Croxon MA, Arena ET, Finlay BB. 2011. A comprehensive study of the contribution of *Salmonella enterica serovar Typhimurium* SPI2 effectors to bacterial colonization, survival, and replication in typhoid fever, macrophage, and epithelial cell infection models. Virulence 2:208–216. [http://dx.doi.org/10.4161/viru.2.3.15894](http://dx.doi.org/10.4161/viru.2.3.15894).

5. Nishino K, Latifi T, Greenman EA. 2006. Virulence and drug resistance roles of multidrug efflux systems of *Salmonella enterica serovar Typhimurium*. Mol Microbiol 59:126–141. [http://dx.doi.org/10.1111/j.1365-2958.2005.04940.x](http://dx.doi.org/10.1111/j.1365-2958.2005.04940.x).

6. Blair JMA, Richmond GE, Piddock LJ. 2014. Multidrug efflux pumps in Gram-negative bacteria and their role in antibiotic resistance. Future Microbiol 9:1165–1177. [http://dx.doi.org/10.2217/fmb.14.66](http://dx.doi.org/10.2217/fmb.14.66).
Bacterial multidrug efflux pumps: much more than antibiotic resistance determinants. Microorganisms 4:14. http://dx.doi.org/10.3390/microorganisms4010014.

Nikaido E, Yamaguchi A, Nishino K. 2000. AcrD of Escherichia coli is an aminoglycoside efflux pump. J Bacteriol 182:1754–1756. http://dx.doi.org/10.1128/JB.182.6.1754-1756.2000.

Baugh S, Ekanayaka AS, Piddock LJ, Webber MA. 2013. Regulation of acrAB by tolC in Escherichia coli serovar Typhimurium strains in impaired ability to form a biofilm. J Antimicrob Chemother 68:2409–2417. http://dx.doi.org/10.1093/jac/dks228.

Blanco P, Hernando-Amado S, Reales-Calderon JA, Coron C, Lira F, Alcalde-Rico M, Bernstein A, Sanchez MB, Martinez JL. 2016. Bacterial multidrug efflux pumps: much more than antibiotic resistance determinants. Microorganisms 4:14. http://dx.doi.org/10.3390/microorganisms4010014.

Nikaido E, Yamaguchi A, Nishino K. 2008. AcrAB multidrug efflux pump regulation in Salmonella enterica serovar Typhimurium by RamA in response to environmental signals. J Biol Chem 283:24245–24253. http://dx.doi.org/10.1074/jbc.M804544200.

Baugh S, Ekanayaka AS, Piddock LJ, Webber MA. 2012. Loss of or inhibition of all multidrug resistance efflux pumps of Salmonella enterica serovar Typhimurium results in impaired ability to form a biofilm. J Antimicrob Chemother 67:2409–2417. http://dx.doi.org/10.1093/jac/dks228.

Usui M, Asai T, Sato S. 2011. Low expression of AcrB in the deoxycholate-sensitive strains of Salmonella enterica subspecies enterica serovar Pullorum. Microbiol Immunol 55:366–368. http://dx.doi.org/10.1111/j.1348-0421.2011.00321.x.

Baucheron S, Mouline C, Praud K, Chaslus-Dancla E, Cloeckaert A, Giraud E. 2014. Bile-mediated activation of the acrAB and tolC multidrug efflux genes occurs mainly through transcriptional derepression of RamA in Salmonella enterica serovar Typhimurium. J Antimicrob Chemother 69:2400–2406. http://dx.doi.org/10.1093/jac/dku140.

Thanassi DG, Cheng LW, Nikaido H. 1997. Active efflux of bile salts by Escherichia coli. J Bacteriol 179:2512–2518.

Usui M, Asai T, Sato S. 2011. Low expression of AcrB in the deoxycholate-sensitive strains of Salmonella enterica subspecies enterica serovar Pullorum. Microbiol Immunol 55:366–368. http://dx.doi.org/10.1111/j.1348-0421.2011.00321.x.

Baucheron S, Mouline C, Praud K, Chaslus-Dancla E, Cloeckaert A. 2005. TolC but not AcrB is essential for multidrug-resistant Salmonella enterica serotype Typhimurium colonization of chicks. J Antimicrob Chemother 55:707–712. http://dx.doi.org/10.1093/jac/dki091.

Virlogeux-Payant I, Baucheron S, Pelet J, Trottier J, Bottreau E, Velge P, Cloeckaert A. 2006. TolC, but not AcrB, is involved in the invasiveness of multidrug-resistant Salmonella enterica serovar Typhimurium via increasing type III secretion system-1 expression. Int J Med Microbiol 298:561–569. http://dx.doi.org/10.1016/j.ijmm.2007.12.006.

Bueel C, Grosse C, Taudte N, Scherer J, Wesenberg D, Krauss GJ, Nies DH, Grass G. 2005. TolC is involved in enterobactin efflux across the outer membrane of Escherichia coli. J Bacteriol 187:6701–6707. http://dx.doi.org/10.1128/JB.187.19.6701-6707.2005.

Ruiz C, Levy SB. 2014. Regulation of acrAB expression by cellular metabolites in Escherichia coli. J Antimicrob Chemother 69:390–399. http://dx.doi.org/10.1093/jac/dkt352.

Horiyama T, Nishino K. 2014. AcrB, AcrD, and MdtABC multidrug efflux systems are involved in enterobactin export in Escherichia coli. PLoS One 9:e108642. http://dx.doi.org/10.1371/journal.pone.0108642.

Webber MA, Bailey MA, Blair JMA, Morgan E, Stevens MP, Hinton JCD, Ivens A, Wain J, Piddock LJ. 2009. The global consequence of disruption of the AcrAB-ToLC efflux pump in Salmonella enterica includes reduced expression of SPI-1 and other attributes required to infect the host. J Bacteriol 191:4276–4285. http://dx.doi.org/10.1128/JB.00363-09.

Hirakawa H, Nishino K, Hirata T, Yamaguchi A. 2003. Comparative studies of drug resistance mediated by overexpression of response regulators of two-component signal transduction systems in Escherichia coli. J Bacteriol 185:1851–1856. http://dx.doi.org/10.1128/JB.185.6.1851-1856.2003.

Appia-Ayme C, Patrick E, Sullivan MJ, Alston MJ, Field SJ, AbouMun N, Anjum MF, Rowley G. 2011. Novel inducers of the envelope stress response BaeSR in Salmonella typhimurium: BaeS is critically required for turgor state disposal. PLoS One 6:e23713. http://dx.doi.org/10.1371/journal.pone.0023713.

Rosner JL, Martin RG. 2013. Reduction of cellular Stress by TolC-dependent efflux pumps in Escherichia coli indicated by BaeSR and CpxARP activation of spy in efflux mutants. J Bacteriol 195:1042–1050. http://dx.doi.org/10.1128/JB.01996-12.

Hirakawa H, Inazumi Y, Masaki T, Hirata R, Yamaguchi A. 2005. Indole induces the expression of multidrug exporter genes in Escherichia coli. Mol Microbiol 55:1113–1126. http://dx.doi.org/10.1111/j.1365-2958.2004.04449.x.

Hu WS, Chen HW, Zhang YR, Huang CY, Shen CF. 2011. The expression of outer membrane proteins STM1530 and OmpD, which are influenced by the CpxARP and BaeSR two-component systems, play important roles in the down-regulation of outer membrane efflux systems of Salmonella enterica serovar Typhimurium. Antimicrob Agents Chemother 55:3829–3837. http://dx.doi.org/10.1128/AAC.00216-11.

Hautefort I, Proença MJ, Hinton JCD. 2003. Single-copy green fluorescent protein gene fusions allow accurate measurement of Salmonella gene expression in vitro and during infection of mammalian cells. Appl Environ Microbiol 69:7480–7491. http://dx.doi.org/10.1128/AEM.69.12.7480-7491.2003.

Sturm A, Heinemann M, Arnoldini M, Benecke A, Ackermann M, Benz M, Dormann J, Hardt W-D. 2011. The cost of virulence: retarded growth of Salmonella typhimurium cells expressing type III secretion system. PLoS Pathog 7:e1002143. http://dx.doi.org/10.1371/journal.ppat.1002143.

Clark L, Perrett CA, Malt L, Harward C, Humphrey S, Jesupon KA, Martinez-Aruguio I, Carney LJ, La Rionge RM, Humphrey TJ, Jesupon KA. 2011. Differences in Salmonella enterica serovar Typhimurium strain invasiveness are associated with heterogeneity in SPI-1 gene expression. Microbiology 157:2072–2083. http://dx.doi.org/10.1099/mic.0.048494-0.

Blair JM, Richmond GE, Bailey AM, Ivens A, Piddock LJ. 2013. Choice of bacterial growth medium alters the transcriptome and phenotype of Salmonella enterica serovar Typhimurium. PLoS One 8:e63912. http://dx.doi.org/10.1371/journal.pone.0063912.

Barak R, Eisenbach M. 1992. Fumarate or a fumarate metabolite restores switching ability to rotating flagella of bacterial envelopes. J Bacteriol 174:643–645.

Barak R, Giebel I, Eisenbach M. 1996. The specificity of fumarate as a switching factor of the bacterial flagellar motor. Mol Microbiol 19:139–144. http://dx.doi.org/10.1046/j.1365-2958.1996.365889.x.

Prasad K, Caplan SR, Eisenbach M. 1998. Fumarate modulates bacterial flagellar rotation by lowering the free energy difference between the clockwise and counterclockwise states of the motor. J Mol Biol 280:821–828. http://dx.doi.org/10.1006/jmbi.1998.2043.

Condell O, Power KA, Händler K, Finn S, Sheridan A, Sergeant K, Renaut J, Burgess CM, Hinton JC, Nally JE, Fanning S. 2014. Comparative analysis of Salmonella susceptibility and tolerance to the biocide...
chlorhexidine identifies a complex cellular defense network. Front Microbiol 5:373. http://dx.doi.org/10.3389/fmicb.2014.00373.

42. Ahn S, Jung J, Jang IA, Madsen EL, Park W. 2016. Role of glyoxylate shunt in oxidative stress response. J Biol Chem 291:11928–11938. http://dx.doi.org/10.1074/jbc.M115.708149.

43. Moreau PL. 2007. The lysine decarboxylase CadA protects Escherichia coli starved of phosphate against fermentation acids. J Bacteriol 189:2249–2261. http://dx.doi.org/10.1128/JB.01306-06.

44. Abernathy J, Corkill C, Hinojosa C, Li X, Zhou H. 2013. Deletions in the pyruvate pathway of Salmonella typhimurium alter SPI1-mediated gene expression and infectivity. J Anim Sci Biotechnol 45: http://dx.doi.org/10.1186/2049-1891-4-5.

45. Feldheim DA, Chin AM, Nierva CT, Feucht BU, Cao YW, Xu YF, Sutrina SL, Saier MH. 1990. Physiological consequences of the complete loss of phosphoril-transfer proteins HPr and FPr of the phosphoenolpyruvate:sugar phosphotransferase system and analysis of fructose (tru) operon expression in Salmonella typhimurium. J Bacteriol 172:5459–5469.

46. Blazy DL, Burns RO. 1984. Regulation of Salmonella typhimurium ilvC genes. J Bacteriol 159:951–957.

47. Rowley G, Hensen D, Felgate H, Arkenberg A, Appia-Ayme C, Prior K, Harrington C, Field SJ, Butt JN, Baggs E, Richardson DJ. 2012. Resolving the contributions of the membrane-bound and periplasmic nitrate reductase systems to nitric oxide and nitrous oxide production in Salmonella enterica serovar Typhimurium. Biochem J 441:755–762. http://dx.doi.org/10.1042/bj3040321.

48. Lu W, Schwarzer NJ, Du J, Gerbig-Smentek E, Andrade SLA, Einsle O. 2012. Structural and functional characterization of the nitrite channel NirC from Salmonella typhimurium. Proc Natl Acad Sci U S A 109:18395–18400. http://dx.doi.org/10.1073/pnas.120793109.

49. Rycrova S, Hatahet I, Fendler K, Michel H. 2012. The nitrite transport protein NirC from Salmonella enterica is a nitrite/proton antiporter. Biochim Biophys Acta 1818:1342–1350. http://dx.doi.org/10.1016/j.bbamem.2012.02.004.

50. Das P, Lahiri A, Lahiri A, Chakravortty D. 2009. Novel role of the nitrite transporter NirC in Salmonella pathogenesis: SP2-dependent suppression of inducible nitric oxide synthase in activated macrophages. Microbiology 155:2476–2489. http://dx.doi.org/10.1099/mic.0.029611-0.

51. Ferreira RB, Buckner MM, Finlay BB. 2012. Genome plasticity in Salmonella enterica and its relevance to host-pathogen interactions, pp 84–102. In Hacker J, Dobrindt U, Kurth R (ed), Genome plasticity and evolution. Infection and Pathogenesis. Springer: pp 84–102. In Hacker J, Dobrindt U, Kurth R (ed), Genome plasticity and evolution. Infection and Pathogenesis. Springer.

52. Buckley AM, Webber MA, Cooles S, Randall LP, La Ragione RM, Woodward LJ, Piddock LJV. 2006. The AccAB-ToLC efflux system of Salmonella enterica and its relevance to host-pathogen interactions, pp 84–102. In Hacker J, Dobrindt U, Kurth R (ed), Genome plasticity and evolution. Infection and Pathogenesis. Springer.

53. Karp PD, Paley S, Romero P. 2002. The Pathway Tools software. Bioinformatics 18:suppl_1:S225–S232. http://dx.doi.org/10.1093/bioinformatics/18.suppl_1.S225.

54. Preuss LC, Andersen SK, Menendez A, Arena ET, Han J, Ferreira BB, Borchers CH, Finlay BB. 2011. Metabolomics reveals phospholipids as important nutrient sources during Salmonella growth in bile in vitro and in vivo. J Bacteriol 193:4719–4725. http://dx.doi.org/10.1128/JB.05132-11.