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[▽][†]

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The mechanisms by which RND pumps contribute to pathogenicity are currently not understood. Using the AcrAB-TolC system as a paradigm multidrug-resistant efflux pump and *Salmonella enterica* serovar Typhimurium as a model pathogen, we have demonstrated that AcrA, AcrB, and TolC are each required for efficient adhesion to and invasion of epithelial cells and macrophages by *Salmonella* in vitro. In addition, AcrB and TolC are necessary for *Salmonella* to colonize poultry. Mutants lacking *acrA*, *acrB*, or *tolC* showed differential expression of major operons and proteins involved in pathogenesis. These included chemotaxis and motility genes, including *cheWY* and *flgLMK* and 14 *Salmonella* pathogenicity island (SPI)-1-encoded type III secretion system genes, including *sopE*, and associated effector proteins. Reverse transcription-PCR confirmed these data for identical mutants in two other *S.* Typhimurium backgrounds. Western blotting showed reduced production of SipA, SipB, and SipC. The absence of AcrB or TolC also caused widespread repression of chemotaxis and motility genes in these mutants, and for *acrB*::*aph*, this was associated with decreased motility. For mutants lacking a functional *acrA* or *acrB* gene, the *nap* and *nir* operons were repressed, and both mutants grew poorly in anaerobic conditions. All phenotypes were restored to that of the wild type by *trans*-complementation with the wild-type allele of the respective inactivated gene. These data explain how mutants lacking a component of AcrAB-TolC are attenuated and that this phenotype is a result of decreased expression of numerous genes encoding proteins involved in pathogenicity. The link between antibiotic resistance and pathogenicity establishes the AcrAB-TolC system as fundamental to the biology of *Salmonella*.

The AcrAB-TolC efflux system is a tripartite complex and is a member of the resistance-nodulation-division (RND) family. It is considered the major efflux system of members of the family *Enterobacteriaceae*. In *Escherichia coli* and *Salmonella enterica*, AcrAB-TolC confers innate resistance to a wide range of toxic substances, including antibiotics, dyes, disinfectants (biocides), and detergents (5, 8, 13, 26, 27, 29). An intact AcrAB-TolC system is also required for the development of high-level, clinically relevant, resistance to numerous agents, including the fluoroquinolones (11, 30, 38). Clinical isolates of multidrug-resistant (MDR) *E. coli* and *S. enterica* commonly overproduce AcrAB-TolC (6, 18, 35, 46, 47). Homologues of AcrAB-TolC found in pseudomonads, *Campylobacter* spp., and *Borrelia burgdorferi*, also confer innate MDR and, when overproduced, clinically relevant levels of antibiotic resistance.

In addition to exporting antibiotics, RND efflux pumps can confer innate resistance to natural substances produced by the host, including bile, hormones, and host defense molecules (33). Efflux pumps of this family are also required for the colonization, and persistence, of bacteria in the host—whether they be plants, animals, or human (34, 35, 45). The mechanisms underpinning this contribution to pathogenesis of RND pumps are not currently well understood. We previously demonstrated that AcrB and TolC are each required for *Salmonella enterica* serovar Typhimurium SL1344 to colonize and persist in poultry (8). One explanation proposed for the attenuation of mutants of *S.* Typhimurium lacking an intact AcrAB-TolC system is that this system is required for growth in the presence of natural detergents, such as the bile found in the chicken intestinal environment. However, this does not explain the inability of the TolC mutant to adhere to (and both the AcrB and TolC mutants to poorly invade) INT-401 and RAW-264.7 tissue culture cell lines in vitro (8). Most recently, we have shown that inactivation of *acrA* also reduces invasion of INT-401 and RAW-264.7 cells by *S.* Typhimurium (Blair et al., personal communication). Nishino et al. (29) found that *S.* Typhimurium strain ATCC 14028 mutants lacking *tolC* or *acrAB* were attenuated in their ability to kill BALB/c mice.

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Control of expression of *acrAB* is complex and can occur at a local level (mediated by the repressor *acrR*) and via global regulators. There is no clear pattern of the hierarchy of regulation of *acrAB*, although the transcriptional regulators MarA and SoxS can affect expression of these genes (36, 41, 49). Although absent in *E. coli* and *Shigella* spp., *S.* Typhimurium and some other members of the family *Enterobacteriaceae* also contain an additional AraC-XylS family regulatory gene, *ramA*. This gene is overproduced in laboratory mutants and MDR clinical and veterinary isolates, whereas overexpression of *marA* and *soxS* is rarely observed (1, 18, 35).

In order to better understand the contribution of RND efflux pumps to pathogenicity, we used the AcrAB-TolC system as a paradigm MDR efflux pump and *S.* Typhimurium as a model pathogen. We investigated the effect of inactivation of *acrA*, *acrB*, or *tolC* on the transcriptome, secretion of virulence-associated proteins, and other virulence-associated attributes of *S.* Typhimurium. Our data indicate that several systems involved in conferring the ability of *S. enterica* to be pathogenic are repressed in mutants in which *acrA*, *acrB*, or *tolC* are inactivated, explaining the attenuation of these strains.

MATERIALS AND METHODS

Bacterial strains. *S.* Typhimurium SL1344 was used as a control strain for all *S. enterica* experiments (51). Mutants of SL1344 lacking a functional *acrA*, *acrB*, or *tolC* gene as a result of insertional activation have been described previously and shown to be nonpolar mutants (8). *E. coli* O78 and its derivative lacking a functional *tolC* were constructed using the method of Datsenko and Wanner (10), as described previously (8). The *acrA::aph* mutant still expresses *acrB*, allowing the results of inactivating each component of AcrAB-TolC to be studied in isolation (Blair, personal communication). The mutant *acrA::aph*, *acrB::aph*, and *tolC::aph* alleles were transferred into 14028s and L3 (35) by P22 transduction as previously described and verified by PCR. Complementation of mutants was as previously described, using low copy number vector pWSK30 (8).

We have previously shown that there are no statistically significant differences between the generation times of all strains during logarithmic phase growth in LB or MOPS (morpholinepropanesulfonic acid) minimal medium, indicating that the growth rate of mutants lacking a functional *acrA*, *acrB*, or *tolC* gene was not impaired or enhanced (see file S1 in the supplemental material) (3). Likewise, total cellular protein (after overnight growth) was not higher in the mutants than in SL1344. No overgrowth was seen for any strain in any media (see file S2 in the supplemental material). The growth kinetics of the *E. coli* O78 *tolC::aph* mutant and the *S.* Typhimurium 14028S Δ*acrAB* mutant (29) were also the same as those of their respective parental strains (data not shown).

Growth kinetics. Growth kinetics of mutants, complemented strains, and SL1344 were investigated by determining absorbance using a spectrophotometer, by viable counts, and by total protein estimation, as described by Yang et al. (52). Growth kinetics in anaerobic conditions were performed using 10-ml volumes of MOPS minimal medium with glucose in sterile universal tubes (Teknova, United States) supplemented with 400 mg/liter histidine overlaid with 10 ml of sterile mineral oil. These tubes were then inoculated with 500 µl of an overnight culture of the test strain and grown at 37°C over 24 h, and then the optical density (OD) was measured. Growth data were obtained in three separate experiments, each containing three technical replicates.

RNA preparation and transcriptional analyses. RNA was prepared from strains after inoculation of 24 ml of defined MOPS minimal medium (Teknova, United States) with 1 ml of overnight culture grown in Luria-Bertani broth. Previously, we established that growth in this medium allows changes in the expression of *acrAB* and *tolC* to be identified easily and more accurately than growth in undefined rich media (4). SPI-1 and SPI-2 were expressed under these conditions. Broths were incubated at 37°C with shaking at 250 rpm. All cultures were grown in sterile 500-ml Erlenmeyer flasks. When the cultures had reached an OD at 600 nm (OD_{600}) of ~0.7 (±0.02), 4 ml of culture was added to 1 ml of 5% phenol and 95% ethanol in a 50-ml falcon tube and incubated on ice for 1 h. Cells were then harvested, and RNA was prepared using the Promega SV40 total RNA preparation kit according to the manufacturer's instructions. The quantity and quality of RNA was determined using a Bioanalyser (Agilent). Transcrip-

tomic experiments used the pan-Salmonella generation IV microarray at the Sanger Genome Campus (Hinxton, United Kingdom). For microarray experiments, three cultures were grown for each strain, and two RNA preparations made from each culture, giving a total of six test and six reference samples (three biological and two technical replicates of each). RNA (25 µg) was used to generate probes labeled with either Cy-3 or Cy-5 using Superscript III (Invitrogen, United Kingdom). For each microarray experiment, wild-type (SL1344) RNA was pooled after quantification to provide a common reference. For each microarray experiment, six slides were hybridized with labeled probes, three with SL1344 labeled with Cy-3 and the test strain labeled with Cy-5 and three dye swaps. Hybridization and scanning were as described previously (12). Results were analyzed using Bioconductor (37). B values (log odds value of >0, and $P <$ 0.05) of <0.05 were taken as significant. All results have been deposited in Array Express.

Comparative reverse transcription (RT)-PCR was used to validate microarray data and determine the expression of genes of interest in different genetic backgrounds, as previously described (13). Primers used to amplify target genes were CheMF, CGCCAATTTCATTACGTCCT; CheMR, TGCCCTGCTTAAC CCATTAG; FlgMF, GCCGTTTTTAATGCTTCGAC; FlgMR, GCATTGACC GTACCTCACCT; NirDF, GTGGCAAAACATCTGCAAAA; NirDR, CGCT ATGGTAAGGACGGAAA; SipAF, CGGTCGTACCGGCTTTATTA; SipAR, CAGACGGCAGAGATTGTGAA; SipCF, GCTAATTTGCTGCTCGGAAC; and SipCR, CCATGGATATGACCCGAATC. 16S rRNA and *ramA* primers were as previously described (13). All PCRs used 30 cycles of 30 s each and an annealing temperature of 51°C. Data were obtained in three separate experiments, each containing three technical replicates. All data were analyzed with Student's *t* test; *P* values of <0.05 were taken as significant.

Protein purification and Western blotting. Western blotting was performed essentially as described previously (24). Strains were grown overnight in Luria-Bertani broth at 25°C, with shaking at 100 rpm. The next morning 4 ml of this culture was used to inoculate 36 ml of sterile Luria-Bertani broth prewarmed to 37°C, and cultures were incubated at 37°C, with shaking at 150 rpm. Once cultures reached an OD_{600} of ~1 (±0.2), the bacteria were harvested by centrifugation, and supernatants were removed and purified in 50-ml falcon tubes using low-protein binding filters (Millipore PVDF, 0.45 µm). To each preparation, trichloroacetic acid was added to a final concentration of 10%, and the preparations were stored on ice for 1 h. After this period, the samples were centrifuged at $10,000 \times g$ for 15 min to pellet proteins. Supernatants were discarded, and proteins were resuspended in 2% sodium dodecyl sulfate and transferred into Eppendorf tubes. Samples were centrifuged again and resuspended in 1 ml of acetone at −70°C overnight. After this incubation the tubes were spun again at $10,000 \times g$ for 10 min, supernatants discarded, and pellets resuspended in 1× LDS loading buffer (Invitrogen, United Kingdom) before being stored at −20°C until required. For preparation of secreted proteins, the cell growth procedure was the same as that followed for RNA extractions prior to the microarray experiments.

For blotting, 5 µl and 0.5 µl of preparations obtained from cells grown in minimal or Luria-Bertani medium were separated, respectively, on 4 to 12% Bis-Tris gels (Invitrogen, United Kingdom). All antibodies used were monoclonal and mouse derived (courtesy of Ed Galyov, Institute for Animal Health) and were used at 1 µg/ml. Magic Mark XP (Invitrogen) was used as a marker in all gels.

Motility assays. The ability of mutants to migrate through (swimming) or across (swarming) semisolid agar was determined by making agar plates based on MOPS minimal medium (Teknova) supplemented with either 0.25, 0.3, or 0.5% (wt/vol) agar. Plates were inoculated by stabbing them with a sterile loop and incubated at 30°C over 5 days, and zones of migration through the agar were measured for each strain daily. Data were obtained in four separate experiments, each containing two technical replicates. All data were analyzed with a Student *t* test; *P* values of <0.05 were taken as significant.

Microarray data accession numbers. The microarray data were deposited in the Array Express database under the following accession numbers: E-MEXP-1832 (SL1344 *acrA::aph*), E-MEXP-1981 (SL1344 *acrB::aph*), and E-MEXP-1982 (SL1344 *tolC::aph*).

RESULTS

Disruption of *acrA* results in differential transcription of 115 genes, including genes encoding proteins involved in pathogenicity. The transcriptomic comparison of L884 (*acrA:: aph*) with SL1344 showed that disruption of *acrA* resulted in

TABLE 1. Genes encoding cell envelope proteins relevant to
antibiotic transport and regulation thereof, anaerobic
metabolism, motility, and chemotaxis, with
significantly different expression
compared with SL1344[a]

Gene/operon or function of encoded protein	Change (fold) in expression relative to SL1344		
	L884 (acrA::aph)	L110 (acrB::aph)	L109 (tolC::aph)
Multidrug transport/ regulation			
acrB	3.22	0.01	0.15
ompC	—	—	—
ompF	—	0.25	0.33
ompR	—	1.55	1.54
ompX	—	0.69	0.68
ramA	—	1,226.41	25.41
rob	—	0.53	—
Anaerobic metabolism			
napA	2.77	0.09	—
napB	—	0.13	—
napC	—	0.14	—
napF	—	0.09	—
narG	2.38	0.01	—
narH	4.69	0.01	—
narI	3.95	0.04	—
narJ	—	0.01	—
narK	3.39	0.01	—
nirB	—	0.01	—
nirC	1.98	0.01	—
nirD	2.62	0.01	—
Motility/chemotaxis			
cheA	2.20	0.07	—
cheM	3.66	0.02	0.39
cheR	—	0.04	—
cheW	2.15	0.01	0.07
cheY	1.96	0.02	0.05
flgC	1.49	0.01	0.13
flgD	1.97	0.01	—
flgE	2.12	0.01	0.11
flgF	2.07	0.05	—
flgG	2.35	0.01	0.13
flgJ	1.83	0.04	—
flgK	1.93	0.01	0.11
flgL	2.10	0.01	0.05
flgM	—	0.01	0.07
flgN	2.12	0.01	0.09
flhD	—	0.61	—
fliA	—	0.01	—
fliD	—	0.13	—
fliS	—	0.07	—
tar	—	0.03	0.09

[a] — indicates no significant change in expression.

TABLE 2. SPI-1 genes and known regulators thereof with
significantly changed expression in L884 (acrA::aph),
L110 (acrB::aph), and L109 (tolC::aph)
compared with SL1344[a]

SPI-1 gene or regulator	Change (fold) in expression relative to SL1344		
	L884 (acrA::aph)	L110 (acrB::aph)	L109 (tolC::aph)
SPI-1 genes			
sitA	—	—	—
avrA	—	0.22	—
STM2868	—	0.45	0.37
orgA (STM 2870)	—	—	0.38
prgH	—	0.06	0.22
prig	—	0.05	0.23
prgJ	—	0.03	0.05
prgK	—	0.13	—
sicP	—	0.42	—
iacP	—	0.32	—
sipA	—	0.08	0.18
sipD	—	0.07	0.25
sipC	—	0.02	0.14
sipB	—	0.02	0.10
spaO	—	0.28	0.30
invJ	—	—	0.12
invB	—	0.03	0.10
Regulators of SPI-1			
hilC	—	0.37	0.35
invF	—	0.10	0.27
fis	—	5.01	—
rtsA	—	0.41	—

[a] — indicates no significant change in expression.

significant (log odds value of >0 and $P < 0.05$) altered expression of 115 genes (3% of the SL1344 genome) (see file S3 in the supplemental material); 54 genes (47%) were increased in expression relative to SL1344, and 61 (53%) were decreased. The gene with the greatest increase (6.7-fold) was *lysC*, responsible for initiation of lysine biosynthesis. Compared to the parental strain, SL1344, there was increased expression of genes that encode proteins important to *Salmonella* infecting and/or colonizing its host. These included chemotaxis genes, flagellum genes, and anaerobic metabolism genes (Table 1). There was also decreased expression of genes that encode

proteins involved in pathogenicity. These included several genes within SPI-2 and genes involved in anaerobic respiration (Table 2). RT-PCR of six genes representing the various clusters confirmed the pattern of significantly differential gene expressions revealed in the microarrays, although the changes revealed by RT-PCR were significantly lower than those indicated by the microarray data in some cases (Fig. 1). Furthermore, RT-PCR revealed that when the mutation in *acrA* was complemented in *trans* (SL1344 *acrA::aph*, pWKS30*acrA*) expression of these genes was the same as that in SL1344 (Fig. 2).

The transcriptomic data were also interrogated for expression of genes encoding efflux or membrane proteins that could compensate for the loss of AcrA. Expression of homologues of *acrA* (*acrE*, *mdtA*, *macA*, *aaeA*, STM0352, and STM0818); *acrB* (*acrD*, *acrF*, *mdtB*, *mdtC*, and STM0351); genes encoding the outer membrane porin proteins, OmpC and OmpF; and those encoding members of the OMP85 family encoding important outer membrane proteins YfgL, YfiO, NlpB, and SmpA (48) was not *acrA* dependent.

Disruption of *acrB* resulted in differential expressions of 569 genes, including genes encoding proteins involved in pathogenicity. Transcriptomic comparison of L110 (*acrB::aph*) and SL1344 showed that disruption of *acrB* caused significant (log odds value of >0 and $P < 0.05$) altered expression of 569 genes (13% of the genome; see file S4 in the supplemental material); 203 genes (36%) were increased in response to *acrB* inactivation. Interestingly, there was relatively little overlap (Fig. 3) between the AcrA- and AcrB-dependent genes which showed significantly increased expression, with only 9 of the 203 (4%)
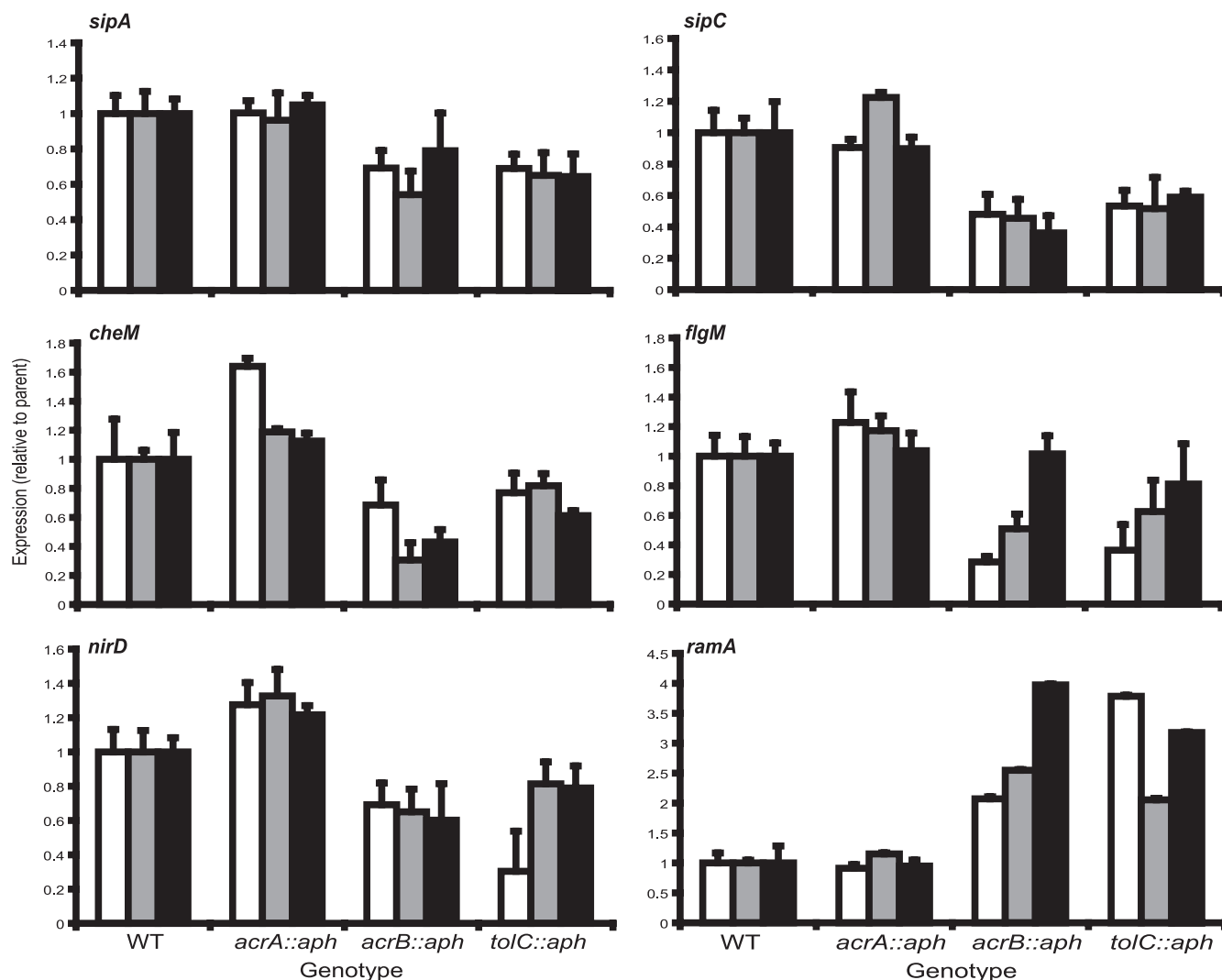
FIG. 1. Validation of transcriptome data. Expression of *sipA*, *sipC*, *cheM*, *flgM*, *nirD*, and *ramA*, as measured by RT-PCR. Open bars indicate SL1344 and mutants thereof; gray bars indicate 14028S and mutants thereof; and black bars indicate L3 and mutants thereof. WT, wild type.

genes being both AcrA and AcrB dependent. The gene with the greatest AcrB-dependent increase in expression (1,200-fold) was *ramA*, previously associated with MDR in *S. enterica* and recently shown to bind upstream of *acrAB* and *tolC* (26). Eight of the 20 most highly AcrB-dependent genes were putative or hypothetical genes without known functions. The expression of the global regulator of transcription, *fis*, was also increased (Table 1).

The expression of 366 (64%) genes was significantly decreased as a result of *acrB* inactivation. Again, there was little overlap with the AcrA-dependent genes; only 16 of the 366 (4%) genes were significantly decreased in expression when *acrA* or *acrB* was inactivated. Compared with SL1344, decreased expression of several clusters of genes that encode proteins involved in pathogenicity was seen with the *acrB* mutant. These genes included *sipABCD*, *invBJ*, and *prgHJI*; the effector genes encoding the proteins SopE and SopE2; genes involved in anaerobic respiration, *napABC*, *napF*, *narGHIJK*, and *nirBC*; genes involved in chemotaxis and motility, including *flgCDEFGJLMLN*, *cheRMWY*, and *tar*; and adhesion genes

fimA, *fimC*, and *fimI*. RT-PCR of representative genes of these clusters confirmed the gene expression changes seen in the microarray experiments. Complementation of *acrB* in *trans* restored gene expression to levels similar to those seen for the parental strain SL1344 (Fig. 2). In contrast to L884 (*acrA*:: *aph*), there was no differential expression of genes in SPI-2 in L110 (*acrB*::*aph*). In addition, while there was differential expression of anaerobic respiration genes in both the AcrA and the AcrB mutants, expression was increased in the former and decreased in the latter.

Inactivation of *acrB* had no effect on the expression of the genes encoding the most homologous transporters to AcrB, AcrD, and AcrF or of the genes encoding members of the OMP85 family. Expression of genes encoding OmpF and OmpX was decreased 4-fold and 1.4-fold, respectively. Of the genes encoding periplasmic stress proteins, only expression of *ppiB* was decreased. Of the genes encoding general stress proteins, *cpx* expression was decreased and σ^E (*rpoE*) was expressed considerably less than it was in SL1344.
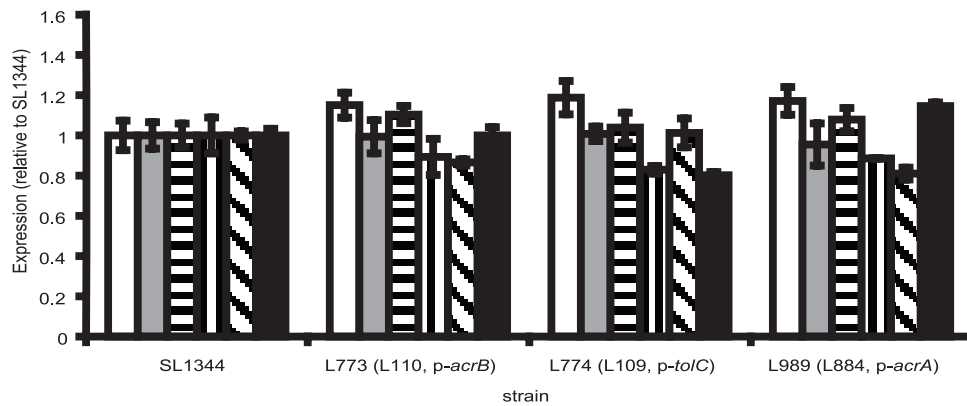
FIG. 2. Complementation of expression of genes altered in mutants. Expression of *ramA* (open boxes), *cheM* (gray boxes), *nirD* (boxes with horizontal lines), *sipC* (boxes with vertical lines), *flgM* (boxes with diagonal lines), *sipA* (black boxes) was measured in SL1344 and L773 (L110 pKSW30*acrB*), L774 (L109 pKSW30*tolC*), and L989 (L884 pKSW30*acrA*). Expression was measured by comparative RT-PCR, and results are shown as change relative to SL1344.

Loss of *tolC* resulted in differential expression of 171 genes, including genes encoding proteins involved in pathogenicity. Far fewer genes were TolC dependent than were AcrB dependent, with altered expression of 171 genes (4% of genome) significantly (log odds value of >0 and *P* < 0.05) different from those in SL1344 (see file S5 in the supplemental material). Fifty-nine genes (35%) showed increased expression in L109 (*tolC*::*aph*). As found for the *acrB* disruptant, the transcriptional activator *ramA* was overexpressed in SL1344 *tolC*::*aph*. Of the 59 genes with increased expression, 27 have not been annotated or have a hypothetical function. Thirty-seven of the 59 (63%) genes upregulated in L109 (*tolC*::*aph*) were also

AcrB dependent for expression. However, there was only one gene dependent upon TolC and AcrA, SL1968, which encodes a putative prophage protein specific to SL1344.

There was significantly (log odds value of >0 and *P* < 0.05) decreased expression of 112 genes after disruption of *tolC*. Of these, 96 were also significantly decreased in expression in L110 (*acrB*::*aph*). The TolC- and AcrB-dependent genes included chemotaxis and motility genes, including *cheWY* and *flgLMK*, and 14 SPI-1 genes, including *sopE* and *sopE2* (Table 1). There was no altered expression of genes in SPI-2. Four of the 112 (4%) TolC-dependent genes were also significantly decreased in expression in L884 (*acrA*::*aph*). Expression of
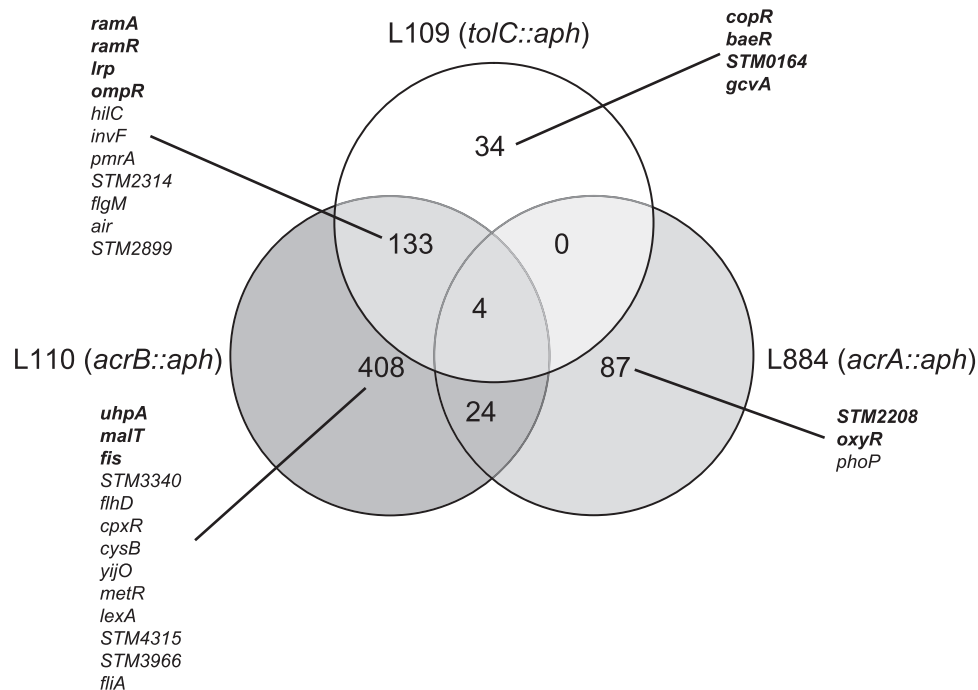

FIG. 3. Comparison of transcriptomes of the *acrA*, *acrB*, and *tolC* mutants. Venn diagram showing overlap of genes with significantly altered (increased or decreased) expression in L884 (*acrA*::*aph*), L110 (*acrB*::*aph*), and L109 (*tolC*::*aph*) compared with SL1344. Regulators with altered expression in each group are indicated. Bold type indicates genes with increased expression; lightface type those with decreased expression.

acrB was significantly reduced in L109 (*tolC*::*aph*), whereas expression of *acrA* was unaffected. The expression of genes encoding other annotated or putative outer membrane efflux proteins (including genes encoding homologues of TolC) and the OMP85 family of *S.* Typhimurium was the same as in SL1344. However, expression of *ompF* and *ompX* was TolC dependent.

No gene was expressed at significantly higher levels in all three mutants, and only four genes in all three mutants were expressed at significantly lower levels than those of SL1344. These were *yaeE* (an ABC transporter permease), STM1698 (a hypothetical protein), *dmsB* (dimethyl sulfoxide reductase), and *glpB* (an anaerobic glycerol hydrogenase). These data indicate that inactivation of *acrA*, *acrB*, and *tolC* has very different effects on the transcriptome of *S.* Typhimurium.

Expression of known regulators, including those of SPI-1, is altered as a result of inactivation of *acrB* or *tolC*. As SPI-1 gene expression was decreased in the AcrB and TolC mutants, the transcriptomic data were interrogated for expression of various genes known to influence regulation of SPI-1 (Table 1). Expression of the major regulator of SPI-1, *hilA*, was not significantly affected in any of the mutants. The major activators of SPI-1 transcription, *invF* and *hilC*, were significantly downregulated in both L110 (*acrB*::*aph*) and L109 (*tolC*::*aph*). The expression of *fis*, which encodes a DNA binding protein known to positively regulate expression of *hilA* and *invF* (25), was increased in L110 (*acrB*::*aph*) only.

A variety of genes are known to be able to influence expression of *acrAB* and *tolC*. Of these, there was no AcrA-, AcrB- or TolC-dependent expression of *acrR*, *marRA*, or *soxRS*. However, expression of *rob* was decreased in the AcrB mutant. *ramA* and *ompR* were overexpressed in response to inactivation of *acrB* or *tolC*.

Expression of the flagellum anti-sigma factor *flgM* was decreased in L110 (*acrB*::*aph*). *flhD* expression was also decreased in L110 (*acrB*::*aph*).

Expression changes seen after inactivation of components of AcrAB-TolC are similar in different strains of *S.* Typhimurium. To confirm the accuracy of the transcriptomic data and that the expression changes seen as a result of disruption of AcrAB-TolC were not specific to the SL1344 background, the mutant *acrA*, *acrB*, and *tolC* alleles were transduced from L884 (*acrA*::*aph*), L110 (*acrB*::*aph*), and L109 (*tolC*::*aph*) into two other wild-type strains of serovar Typhimurium, 14028s and L3 (human isolate). Comparative RT-PCR was used to measure expression of *sipA*, *sipC*, *cheM*, *flgM*, *nirD*, and *ramA* in the nine mutants compared with that in the respective parental strains. Irrespective of the strain of *S.* Typhimurium, disruption of *acrA*, *acrB*, or *tolC* conferred altered expression of these six genes and mirrored the changes of expression observed in the transcriptomic data sets. These data indicate that data obtained for SL1344 are typical of this serovar (Fig. 1). Furthermore complementation of the mutant *acrA*, *acrB*, and *tolC* alleles in the different strain backgrounds restored expression of all genes tested to levels not significantly different from that in SL1344 (Fig. 2).

Secretion of SipA, SipB, and SipC is reduced in mutants lacking components of AcrAB-TolC. One striking finding of the transcriptional analyses was that expression of genes known to be involved in pathogenesis was significantly decreased when
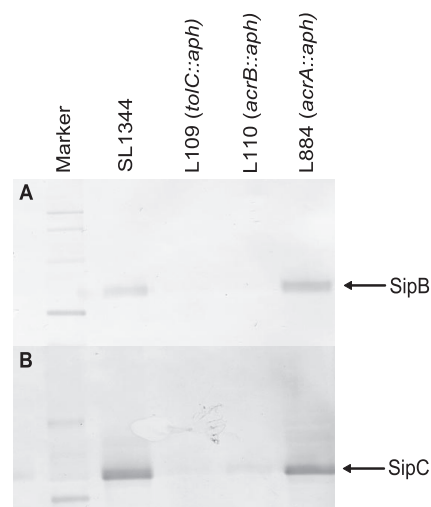

FIG. 4. Production of SPI-1 proteins. Western blot of SipB and SipC of protein supernatants prepared from L884 (*acrA*::*aph*), L110 (*acrB*::*aph*), and L109 (*tolC*::*aph*) grown in minimal media.

acrB or *tolC* was inactivated. Therefore, production of the secreted SPI-1 proteins, SipA, SipB, and SipC, by each mutant was analyzed by Western blotting. Culture supernatants were prepared from cells grown in minimal medium in order to replicate the growth conditions of the transcriptomic experiments. Data from Western blotting were in agreement with the transcriptomic data, with significantly reduced amounts of SipB and SipC detected in both L109 (*tolC*::*aph*) and L110 (*acrB*::*aph*). Consistent with the transcriptomic data, secretion of these proteins was not significantly reduced in L884 (*acrA*::*aph*) (Fig. 4). After growth of L109 (*tolC*::*aph*) in minimal medium, too little of SipA was produced to be detected; however, significantly less SipA was observed with extracts from cells grown to late logarithmic phase in Luria-Bertani medium compared to SipA in SL1344 (data not shown).

Mutants lacking *acrB* showed reduced motility. As genes involved in chemotaxis and motility were decreased in expression in both L110 (*acrB*::*aph*) and L109 (*tolC*::*aph*), the ability of each strain to migrate through or over semisolid agar was evaluated. All strains retained the ability to "swim" and "swarm." Only L110 (*acrB*::*aph*) showed a significant ($P <$ 0.05) loss in motility. This strain was significantly less motile than SL1344 on minimal agar for all three concentrations tested, indicating a reduced ability to both swim and swarm (Fig. 5). Electron micrographs indicated that all mutants have morphologies similar to that of SL1344, but fewer flagella were visible in preparations of L110 (*acrB*::*aph*) (data not shown).

L110 (*acrB*::*aph*) and L884 (*acrA*::*aph*) are compromised in their ability to grow anaerobically. Expression of genes in the *nir*, *nar*, and *nap* operons encoding genes involved in anaerobic respiration was reduced in L110 (*acrB*::*aph*) and increased in L884 (*acrA*::*aph*). However, the ability of both these mutants to grow in anaerobic conditions was impaired compared to the ability of SL1344 (Fig. 6) after 8 and 24 h. L109 (*tolC*::*aph*) did not show altered expression of genes involved in anaerobic respiration, and its anaerobic growth was not significantly different from SL1344 under anaerobic conditions.
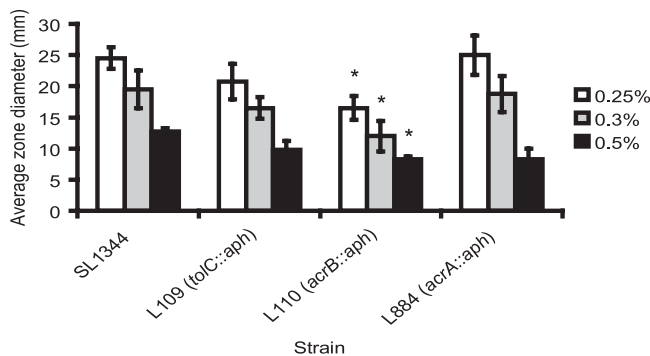
FIG. 5. Motility of mutants. Average zones of motility (± standard deviation) of SL1344, L884 (*acrA::aph*), L110 (*acrB::aph*), and L109 (*tolC::aph*) in three concentrations of semisolid minimal medium agar. Asterisks indicate statistically significant (*P* < 0.05) differences.

FIG. 6. Growth kinetics of mutants. Growth (± standard deviation) of L884 (*acrA::aph*), L110 (*acrB::aph*), and L109 (*tolC::aph*) in anaerobic conditions. Asterisks indicate statistically significant (*P* < 0.05) differences.

Interrogation of published data for the effect of environmental conditions on growth in tissue culture cells upon expression of *acrA* or *tolC*. The AcrAB-TolC system contributes to a variety of phenotypes, including pathogenicity in *S.* Typhimurium, and data presented herein indicate that this may be due to repression of several regulons that influence expression of genes known to be important in pathogenicity. The *acrAB* and *tolC* genes are among the most highly expressed genes of *S.* Typhimurium, and chromatin immunoprecipitation and microarray experiments have shown RNA polymerase bound tightly to the *acrAB* locus (22). Expression of *acrAB* and *tolC* is relatively stable under different environmental conditions in vitro (data not shown). To determine whether expression of *acrA* or *tolC* is regulated during the pathogenesis of *S.* Typhimurium, transcriptomic data previously obtained for SL1344 under diverse conditions (19, 22, 44) were analyzed for changes in expression of *acrA* and *tolC* (*acrB* was not present on the microarray used in these experiments). In LB media, both *acrA* and *tolC* were produced at maximal levels during the early logarithmic phase, and expression of both genes was increased by 4.5-fold at 37°C compared to that at 25°C (31). In LB medium, expression of *acrA* can be induced by polymyxin (2), whereas in the presence of hydrogen peroxide, expression of *acrA* was decreased threefold. Expression of *tolC* was decreased 10-fold after growth in macrophages (J774) (17) and 5-fold after growth in HeLa epithelial cells (19). Neither *acrA* or *tolC* was regulated by the nucleoid-associated proteins H-NS (31), IHF (9, 23), or Fis (20) or the RNA chaperone protein Hfq (40).

A number of regulatory genes are known to be able to influence the expression of genes involved in pathogenicity or defense against host-derived factors of *S.* Typhimurium; these include the two-component *phoPQ* (53) and *pmrAB* (42) systems (involved in sensing the transition to an intracellular location and defense against host peptides) and the regulators of SPI-1, *invF* (9), *hilC* (*sprA*) (14), and *hilA* (43), and of SPI-2, *ssrA* (39), as well as global regulators, including *fis* (7, 50) and *hns* (28). Of these regulatory genes, only one (*invF*) had decreased expression when *acrB* or *tolC* was inactivated (Table 1). In addition, in L110 (*acrB::aph*), there was decreased expression of *pmrA* and *pmrB* compared with that in SL1344. This mutant also had increased expression of *fis*. Analysis of
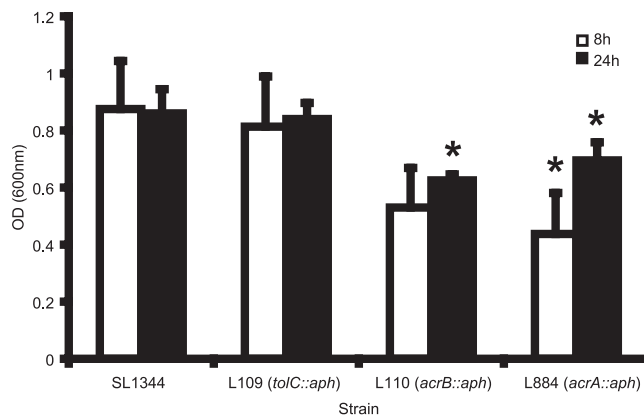
the available transcriptomic data and literature for these systems identified that *phoPQ* is postulated to regulate *tolC* (53), although *phoPQ* expression was unchanged in SL1344 *tolC::aph*. There are currently no data indicating that the other systems directly influence expression of *acrAB* or *tolC*.

DISCUSSION

While it has been shown that inactivation of RND family efflux systems can confer attenuation in the host, there is little evidence of how these systems are involved in pathogenesis. The present study sought to explore the cause of the observed attenuation in mutants of *S. enterica*, each lacking a component of the AcrAB-TolC system, using total genome transcriptional analyses to guide biological experiments.

The transcriptomic experiments in this study revealed that in response to inactivation of *acrA*, *acrB*, or *tolC* there were considerable gene expression changes. These included genes related to the ability of the mutants to infect the host, as well as those that could compensate for loss of AcrA, AcrB, or TolC (i.e., efflux pumps or outer membrane proteins and regulators thereof). The transcriptomic, confirmatory RT-PCRs and Western blotting data demonstrated a general repression of SPI-1 in *S.* Typhimurium, resulting from inactivation of *acrB* or *tolC*, and repression of SPI-2 after inactivation of *acrA*, demonstrating that this phenomenon is not restricted to SL1344.

SPI-1 is required for subversion of the host cells' cytoskeletal machinery by *S.* Typhimurium, which leads to ruffle formation and uptake of *S.* Typhimurium into host cells. A previous study by Morgan et al. (25) used signature-tagged mutagenesis to identify genes required for colonization of chicks and cattle. In their study, the disruption of SPI-1 genes resulted in only a minor defect in the ability of SL1344 to colonize 2-week-old chicks; however, deletion of *sopE2* resulted in a colonization defect. In this transcriptomic study, decreased expression of *sopE2*, as well as SPI-1 genes, was observed with both L109 (*tolC::aph*) and L110 (*acrB::aph*). Other systems are also required during the pathogenesis process; the ability to grow anaerobically was impaired in L110 (*acrB::aph*), which may negatively impact the ability of this mutant to survive in the

gut. Motility is also important in the pathogenesis of *S.* Typhimurium (21), and L110 (*acrB*::*aph*) was less motile than SL1344. Therefore, we postulate that the inability of mutants lacking a functional AcrAB-TolC system to invade tissue culture cells or to colonize chicks is due to decreased expression of specific genes encoding proteins conferring the ability to infect the host; these include the SPI-1 and SPI-2 genes and other genes which contribute to the ability of *S.* Typhimurium to survive in vivo and attach to host cells. It is likely that the observed decreased expression of SPI-1 is mediated, in part, by the decreased expression of *invF* seen with all mutants, as *invF* has been shown to be required for expression of various SPI-1 genes (9). Recently it has been demonstrated (32) that inactivation of the magnesium transporter *corA* in *S.* Typhimurium results in attenuation in the mouse infection model. Deletion of *corA* also resulted in varied changes to the transcriptome of *S.* Typhimurium, including repression of SPI-1, SPI-2, and motility-associated genes. This pattern is similar to the results observed here, although examination of the data reveals no obvious regulatory link between the two studies. It is possible that deletion of *corA* and of *acrAB* and *tolC* perturbs a common but currently unrecognized regulatory network.

Virlogeux-Payant et al. (45) recently reported that inactivation of *tolC* led to decreased expression of SPI-1 genes, which is in agreement with our data. However, in contrast to data presented herein, they reported that inactivation of *acrB* did not lead to a decrease in SPI-1 expression in the isolates they tested. They also previously reported that AcrB is not required for colonization of chickens by *S.* Typhimurium (5). One possible explanation for the difference between these data is that we used SL1344 mutants (this study and reference 8) and Baucheron et al. used various isolates of DT104 and DT204. In order to address this, the mutant alleles of *acrA*, *acrB*, and *tolC* were transduced into two additional strains of *S.* Typhimurium, 14028s and a human clinical isolate, L3; data very similar to those from the SL1344 mutants were obtained. In addition, 14028s *acrAB*::*Cm* administered by the oral route was attenuated in mice (29). These data indicate that the data obtained for SL1344 mutants are not specific to this background alone. The discrepancies seen with DT104 and DT204 do, however, suggest that while TolC would appear to influence expression of SPI-1 and SPI-2 in all strains tested to date, AcrB has a similar effect in some strains but not all. A role for AcrB in pathogenicity is, however, further supported by data showing that homologues of this protein are also required for other bacterial species to colonize and/or infect their host (34).

While there were similarities and differences between the transcriptomes of all three mutants, there was much greater overlap between the transcriptomes of L109 (*tolC*::*aph*) and L110 (*acrB*::*aph*), particularly among those genes with decreased expression. These data may explain similarities in phenotypes between these mutants; however, there are also distinct phenotypic differences. For instance, mutants lacking *tolC* are unable to adhere to cells in tissue culture; in contrast, mutants lacking *acrA* and *acrB* can adhere but are poorly invasive (8). It has been postulated that disruption of AcrAB-TolC and in particular TolC (an important structural component of the cell envelope of the members of *Enterobacteriaceae*) confers membrane instability and that this may confer

the observed attenuation. However, there were no gene expression changes indicative of membrane stress seen with any of the mutants, including no alteration in expression of genes encoding Omp85 family proteins, shown to be required for assembly of numerous outer membrane proteins (48). This may have been anticipated if loss of AcrAB-TolC had a nonspecific effect on membrane stability. It is currently unclear why mutants lacking *tolC* are more attenuated than those lacking *acrA* or *acrB* but is perhaps simply due to the more promiscuous nature of TolC. For instance, TolC interacts with several other proteins (e.g., AcrEF and MacAB), and its loss also impacts upon export of substrates by those systems as well as those of AcrAB. In addition, in *S.* Typhimurium lack of MacAB also confers attenuation (29). When combined with inactivation of *acrAB* and *macAB* the bacterium is severely disabled. AcrA has also been shown to be promiscuous (15, 16) and can interact with AcrD, it is conceivable that homologues of AcrA, such as AcrE, may be similarly able to bind to AcrB and TolC and complement the effects of inactivation of *acrA* to some extent.

We, and others, recently showed that *ramA* regulates expression of *acrB* (12, 31) and that *ramA* itself is regulated by *ramR* (1). Data presented herein indicate that expression of *ramA* is also responsive to inactivation of *acrB* and *tolC* but not *acrA*. It is hypothesized that in L110 (*acrB*::*aph*) and L109 (*tolC*::*aph*), *ramA* expression is increased in response to a lack of expression of the inactivated genes. One explanation for this is that an inducer of *ramA* (either a metabolic product usually exported via AcrAB-TolC or a component of the media used in our studies) is a substrate of AcrAB-TolC and that deletion of *acrB* and *tolC* results in accumulation of this inducer and increased expression of *ramA*.

Although inactivation of *acrA*, *acrB*, or *tolC* conferred gene expression changes upon known regulators of genes that encode proteins involved in infecting the host, the mechanism by which these changes are mediated is unclear. With the exception of PhoPQ, recently shown to be able to regulate *tolC* in *E. coli* (53), there is currently no known regulatory network which includes both *acrB* and *tolC* and regulators of pathogenesis. Some of the conditions known to be relevant to the ability of *S. enterica* to be pathogenic, such as pH and temperature, also influence expression of *acrA* and *tolC*. This may suggest that undescribed regulatory mechanisms that control expression of *acrB* and *tolC* are also involved in controlling genes that confer pathogenicity.

In conclusion, transcriptomic and biological data presented herein indicate that the attenuation of *S.* Typhimurium lacking AcrB or TolC is a result of decreased expression of many genes involved in the pathogenic process, including those required for anaerobic growth, motility, and host cell invasion, particularly SPI-1. These results are likely to be applicable to related species for which RND pumps have also been shown to contribute to pathogenicity, and these results may represent a general effect in which large numbers of genes are dependent on the presence of a functional major efflux system for expression. The link between innate MDR and pathogenicity establishes RND efflux pumps and the AcrAB-TolC system in particular as fundamental to the biology of the organism.

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