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Arginine-Dependent Acid Resistance in *Salmonella enterica* Serovar Typhimurium

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***Salmonella enterica* serovar Typhimurium does not survive a pH 2.5 acid challenge under conditions similar to those used for *Escherichia coli* (J. W. Foster, Nat. Rev. Microbiol. 2:898–907, 2004). Here, we provide evidence that *S. enterica* serovar Typhimurium can display arginine-dependent acid resistance (AR) provided the cells are grown under anoxic conditions and not under the microaerobic conditions used for assessment of AR in *E. coli*. The role of the arginine decarboxylase pathway in *Salmonella* AR was shown by the loss of AR in mutants lacking *adiA*, which encodes arginine decarboxylase; *adiC*, which encodes the arginine-agmatine antiporter; or *adiY*, which encodes an AraC-like regulator. Transcription of *adiA* and *adiC* was found to be dependent on AdiY, anaerobiosis, and acidic pH.**

Acid resistance (AR) in the *Enterobacteriaceae* requires the presence of amino acids in the challenge medium; while *Escherichia coli* and *Shigella flexneri* are classified as AR, it has repeatedly been found that *S. enterica* serovar Typhimurium does not survive a pH 2.5 acid challenge under conditions similar to those used for *E. coli* (8, 9, 12, 15). The AR mechanisms in *E. coli* have been demonstrated by several researchers; they enable this organism to survive at pHs as low as 2.5 (15, 16, 20). The first AR system in *E. coli* (AR 1) is apparent when cells are grown to stationary phase in rich medium and subsequently challenged in EG mineral medium at pH 2.5 (4). The alternative sigma factor RpoS and the global regulatory protein cyclic AMP receptor protein regulate AR 1. Repression of AR 1 by glucose permitted the discovery of the amino acid-dependent AR systems in *E. coli* (15). Cells that have been grown in rich medium, pH 5.5, to stationary phase in the presence of glucose survive dilution in mineral medium, pH 2.5, only in the presence of glutamate (AR 2), arginine (AR 3), and to a lesser extent lysine (AR 4) (4, 13). AR 2 is the most extensively studied mechanism of the three amino acid decarboxylase systems in *E. coli* (5, 9), but it seems to be absent from *S. enterica* serovar Typhimurium (15). The genes for the AR 3 system are present in *E. coli* and *S. enterica* serovar Typhimurium but seem to contribute to AR only in *E. coli*. In *E. coli*, the arginine decarboxylase (*adiA*) and the arginine-agmatine antiporter (*adiC*) are responsible for acid resistance (11). CysB is a positive regulator of *adi* transcription, and, when overexpressed, the AraC-like regulator AdiY is also involved in regulation of *adiA* (19, 22). The AR 4 system is not present in *E. coli*, but in *S. enterica* serovar Typhimurium the lysine decarboxylase (*cadA*) and the lysine-cadaverine antiporter (*cadB*) work in concert to protect this organism at pHs as low as 3.0 (18).

It has generally been recognized that *S. enterica* serovar Typhimurium is not able to survive pH values as low as 2.5 (9, 12, 15). Recently, however, de Jonge et al. (7) showed that *S. enterica* serovar Typhimurium was able to survive in mineral medium at pH 2.5 for a prolonged period of time, and this triggered us to investigate the role of L-arginine in AR 3 in *S. enterica* serovar Typhimurium in more detail.

Arginine-dependent AR in *S. enterica* serovar Typhimurium. AR 3 in stationary-phase *S. enterica* serovar Typhimurium cells grown in LBG was determined as described previously (3). In short, 10 μl of stationary-phase culture was transferred to 10 ml of fresh EG mineral medium at pH 2.5. EG mineral medium consisted of Vogel-Bonner mineral medium E (24), 100 mM MES (morpholineethanesulfonic acid), 10 mM L-arginine, and 4 g/liter of glucose at pH 2.5. Growth of the cells in a 10-ml filled screw-cap culture tube without shaking created anoxic conditions. Without the addition of L-arginine, the number of surviving bacteria decreased by more than 4 log after 1 h at pH 2.5. The amounts of *S. enterica* serovar Typhimurium DT104 strain BAA-188 (2) that survived in challenge medium supplemented with L-arginine were approximately 59.4% and 12.5% after 1 and 2 h, respectively, at pH 2.5 (Fig. 1). Similar results were obtained for the other serovar DT104 strains. The survival rates of *S. enterica* serovar Typhimurium LT2 (10) and UK1 (6) at pH 2.5 were slightly higher, at approximately 83% and 38% after 1 and 2 h, respectively.

The results presented describe for the first time arginine-dependent stationary-phase AR in *S. enterica* serovar Typhimurium under conditions similar to those used for *E. coli*. All seven *S. enterica* serovar Typhimurium strains tested displayed this phenotype, suggesting a common property of *S. enterica* serovar Typhimurium strains. Omission of L-arginine from the challenge medium resulted in an acid-sensitive phenotype in all *S. enterica* serovar Typhimurium strains tested. Our results show that the LT2 strain displays AR similar to those of the *S. enterica* serovar Typhimurium DT104 strains, which were

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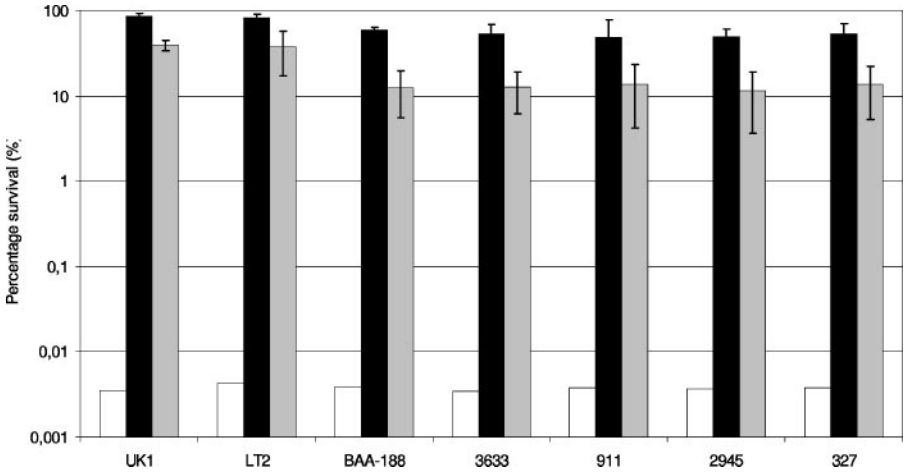

FIG. 1. Arginine-dependent AR of stationary-phase *S. enterica* serovar Typhimurium cells. Stationary-phase cells grown in LBG at pH 5 were challenged in EG mineral medium, pH 2.5, at an initial concentration of approximately 5×10^5 CFU/ml. Percent survival is depicted as follows: open bars, survival after 1 h without L-arginine; solid bars, survival with L-arginine after 1 h; gray bars, survival with L-arginine after 2 h. All experiments were performed twice in triplicate. The detection limit of the experiment was 5×10^2 CFU/ml.

shown in a previous study by Berk et al. (3) to contain wild-type *rpoS*. This indicates, as suggested by Park et al. (17), that RpoS has no major role, if any role at all, in AR 3 in *S. enterica* serovar Typhimurium.

Role of oxygen in arginine-dependent AR *S. enterica* serovar Typhimurium DT104. Previous reports stated that *S. enterica* serovar Typhimurium does not survive pH 2.5 acid challenge under conditions similar to those used for *E. coli* (9, 12, 15). Notably, in those studies cells grown under microaerobic conditions were used. Therefore, we assessed the role of oxygen in more detail in the development of arginine-dependent AR in *Salmonella* during culturing. *S. enterica* serovar Typhimurium DT104 strain BAA-188 was grown to stationary phase in LBG, pH 5, under anoxic, microaerobic, and aerobic conditions and subsequently challenged in mineral medium supplemented with 20 mM L-arginine at pH 2.5 under different oxygen conditions (Table 1). Aerobic cultures (10 ml) were grown in 250-ml Erlenmeyer flasks with shaking at 225 rpm. Cultures grown under microaerobic conditions, the standard conditions used for assessment of *E. coli* AR (15), were obtained by growing the cells in 3 ml of medium in 15- by 150-mm test tubes with shaking at 225 rpm. Analysis of stationary-phase AR under different oxygen conditions revealed that both aerobically and microaerobically grown stationary-phase *S. enterica* serovar Typhimurium cells were unable to survive at pH 2.5, whereas anaerobically grown cells again showed excellent survival. Moreover, the presence of oxygen during the AR test at pH 2.5 resulted in a decreased survival capacity. Under microaerobic challenge conditions, approximately 18.4% and 1.2% of the anaerobically grown cells survived after 1 and 2 h, respectively. Under aerobic conditions, the survival of these cells decreased even further, to approximately 7.8% and 0.3% after 1 and 2 h, respectively.

TABLE 1. Impact of oxygen conditions on arginine-dependent AR of stationary-phase *S. enterica* serovar Typhimurium DT104 strain BAA-188 cells[a]

Growth condition	Challenge condition	% Survival (±SD) at pH 2.5 after:	
		1 h	2 h
Anoxic	Anoxic	78.2 ± 4.7	68.6 ± 1.8
Anoxic	Microaerobic	18.4 ± 1.7	1.2 ± 0.03
Anoxic	Aerobic	7.8 ± 4.8	0.3 ± 0.2
Microaerobic	Anoxic	<0.005	<0.005
Microaerobic	Microaerobic	<0.005	<0.005
Microaerobic	Aerobic	<0.005	<0.005
Aerobic	Anoxic	<0.005	<0.005
Aerobic	Microaerobic	<0.005	<0.005
Aerobic	Aerobic	<0.005	<0.005

[a] Aerobic, microaerobic, and anaerobic stationary-phase cells grown in LBG at pH 5 were challenged in EG mineral medium, pH 2.5, with 20 mM L-arginine at an initial concentration of approximately 1×10^6 CFU/ml under different oxygen conditions. All experiments were performed twice in triplicate. The detection limit was 5×10^2 CFU/ml.

Obviously, the oxygen condition during growth is a critical determinant of the arginine-dependent AR phenotype in *Salmonella*; i.e., only anaerobically grown stationary-phase cells display the AR phenotype. Our results offer an explanation for the lack of AR noted by other researchers, since in their experiments microaerobic growth conditions were used for testing AR at pH 2.5 (9, 12, 15). Culture conditions that resulted in an AR phenotype in *E. coli* did not result in an AR phenotype in *S. enterica* serovar Typhimurium. Apparently, development of arginine-dependent AR in *Salmonella* is strictly dependent on anoxic growth conditions.

Role of the arginine decarboxylase pathway in *S. enterica* serovar Typhimurium DT104 AR. To assess the role of the arginine decarboxylase system in *S. enterica* serovar Typhimurium DT104 AR at pH 2.5, several mutants in the *adi* gene cluster were constructed. Suicide plasmids were constructed by PCR amplification of an internal fragment of the target gene with *Pwo* polymerase (Roche Diagnostics, Almere, The Netherlands) and subsequent insertion in the λ *pir*-dependent plasmid pERFORM-Z (1) by methods described previously (14). Single-crossover transconjugants were selected with zeocin (25 μg/ml ml) and tetracycline (12.5 μg/ml) for counterselection against *E. coli* upon conjugation. PCR and Southern analysis

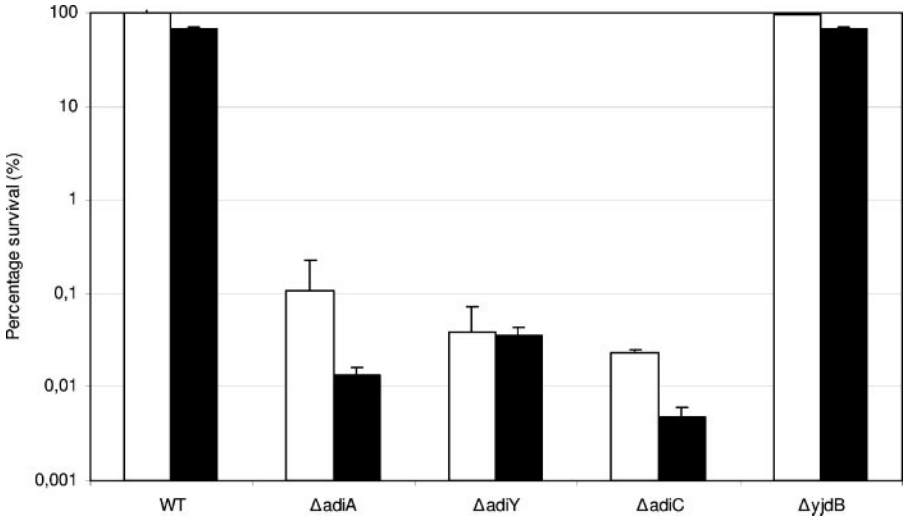

FIG. 2. Arginine-dependent AR of stationary-phase *S. enterica* serovar Typhimurium DT104 strain BAA-188. Stationary-phase cells grown in LBG at pH 5 were challenged in EG mineral medium, pH 2.5, with 20 mM L-arginine at an initial concentration of approximately 5×10^5 CFU/ml. Survival after 1 h (open bars) and 2 h (black bars) is depicted. All experiments were performed twice in triplicate. WT, wild type.

confirmed that the selected strains harbored the inserted antibiotic resistance cassette or plasmid (data not shown). In this way, *S. enterica* serovar Typhimurium DT104 strain BAA-188 *adiA* (arginine decarboxylase), *adiY* (regulator of *adiA*), *yjdB* (putative antiporter in the *adi* gene cluster), and *adiC* (arginine-agmatine antiporter) deletion mutants were obtained.

As demonstrated in Fig. 2, disruption of the arginine decarboxylase gene *adiA* results in an AR of 0.014% after 2 h at pH 2.5, compared to 68.6% for the wild type. A dramatic loss of AR was also observed in the *adiY* (transcriptional regulator) and *adiC* (arginine-agmatine antiporter) deletion mutants, which showed 0.035% and 0.048% survival, respectively, after 2 h at pH 2.5 in challenge medium supplemented with 20 mM L-arginine. Disruption of *yjdB*, encoding a putative antiporter, downstream of *adiC* did not result in loss of the AR phenotype.

The arginine decarboxylase system has been implicated in AR in *E. coli* and *Shigella flexneri* (15). From the results presented here, it is obvious that the arginine decarboxylase system, including the arginine decarboxylase (*adiA*), the AraC-like regulator (*adiY*), and the arginine-agmatine antiporter (*adiC*), confers AR in *S. enterica* serovar Typhimurium.

Expression of *adi* genes in *S. enterica* serovar Typhimurium DT104 strain BAA-188. RNA from mid-logarithmic-phase *S. enterica* serovar Typhimurium DT104 strain BAA-188 cultures grown in LBG was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with RQ1 RNase-free DNase (Promega, Leiden, The Netherlands), as recommended by the supplier. Subsequently the expression of *adiA*, *adiC*, and *adiY* was examined by quantitative PCR (qPCR) using the Bio-Rad MyiQ single-color real-time PCR detection system (Bio-Rad, Veenendaal, The Netherlands) and the iQ SYBR green Supermix kit (Bio-Rad), as recommended by the manufacturer. Primer sets were designed with Beacon Designer, version 4.02 (PREMIER Biosoft International, Palo Alto, CA). After optimization, the mean relative expression values were calculated. Expression of *adiA* and *adiC* was found to be induced by acid and anoxic growth conditions (Table 2). Similar results have been reported for transcription regulation of *adiA* in *E. coli* (11, 21) and *adiC* in *Salmonella* (17). The expression of *adiY* appears to be higher in *Salmonella* grown under aerobic conditions and to not be affected by the pH. It has been reported that the XylS/AraC-like transcriptional regulator AdiY, which is part *adi* gene cluster, acts as a positive regulator of *adiA* in *E. coli* (22). The data presented here reveal that *adiY* is essential for induction of arginine-dependent AR in *Salmonella*. Indeed, qPCR analysis revealed transcription of *adiA* and *adiC* to be reduced significantly in the *adiY* deletion mutant (Table 2). Recently, Gong et al. (11) suggested that AdiY acts as a conditional regulator for *adiA*, as they failed to shown a reduction in *adiA* transcription in an *E. coli adiY* deletion mutant. In *E. coli*, furthermore, the XylS/AraC-like transcriptional regulators EnvY and AppY were shown to stimulate *adiA* transcription (22). In *S. enterica* serovar Typhimurium, however, *adiY* has a unique role in that it is essential in the

TABLE 2. Change in relative gene expression in *S. enterica* serovar Typhimurium DT104 strain BAA-188 as measured by qPCR[a]

Strain genotype	Growth conditions	Fold change in gene expression[b]		
		adiA	*adiY*	*AdiC*
Wild type	Anoxic, pH 7	1.0	1.0	1.0
Wild type	Aerobic, pH 5	8.1	4.7	2.8
Wild type	Microaerobic, pH 5	6.0	2.7	3.0
Wild type	Anoxic, pH 5	95.1	1.0	48.6
Δ*adiA*	Anoxic, pH 5	—	2.9	162.7
Δ*adiY*	Anoxic, pH 5	2.3	—	1.4
Δ*adiC*	Anoxic, pH 5	6.6	1.0	—

[a] RNA for the *tpiA* gene, encoding the triose-phosphate isomerase that converts D-glyceraldehyde 3-phosphate into glycerone phosphate, was used as a housekeeping gene for normalization (23).

[b] Relative gene expression of the wild type under anoxic growth conditions at pH 7 was set at 1.0. The standard deviation of the mean change in the experiment was <25%. —, not determined.

induction of arginine-dependent AR via direct or indirect transcriptional activation of the *adiA* and *adiC* genes.

In conclusion, this study clearly shows that *S. enterica* serovar Typhimurium has an active arginine-dependent AR mechanism (AR 3), permitting survival at pH 2.5. The *adiA*, *adiY*, and *adiC* gene products are critically important in *Salmonella* AR 3, with *adiY* playing a crucial role in the regulation of transcription of *adiA* and *adiC*. Foster recently stated that there was more to the AR 3 system of *Salmonella* than simply finding an active arginine decarboxylase and arginine-agmatine antiporter (9). From our results, however, it is now clear that the AR 3 system contributes to pH 2.5 acid survival only when *Salmonella* is grown anaerobically and not under conditions normally used for assessment of AR in *E. coli*.

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