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aro Mutations in *Salmonella enterica* Cause Defects in Cell Wall and Outer Membrane Integrity▽

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In this study we characterized *aro* mutants of *Salmonella enterica* serovars Enteritidis and Typhimurium, which are frequently used as live oral vaccines. We found that the *aroA*, *aroD*, and *aroC* mutants were sensitive to blood serum, albumen, EDTA, and ovotransferrin, and this defect could be complemented by an appropriate *aro* gene cloned in a plasmid. Subsequent microarray analysis of gene expression in the *aroD* mutant in serovar Typhimurium indicated that the reason for this sensitivity might be the upregulation of *murA*. To confirm this, we artificially overexpressed *murA* from a multicopy plasmid, and this overexpression caused sensitivity of the strain to albumen and EDTA but not to serum and ovotransferrin. We concluded that attenuation of *aro* mutants is caused not only by their inability to synthesize aromatic metabolites but also by their defect in cell wall and outer membrane functions associated with decreased resistance to components of innate immune response.

In the early 1980s it was learned that *Salmonella* mutants auxotrophic for aromatic amino acids have reduced virulence for animals (14). Since that time, mutations in genes coding for the biosynthesis of aromatic amino acids have been used frequently to reduce the virulence of different *Salmonella* sp. strains. *aroA* and *aroD* mutants of *Salmonella enterica* serovar Typhi were successfully tested as a vaccine against human typhoid (31, 32), and the same mutations were used also for the construction of avirulent strains for immunization of different farm animals (5, 10, 22). *aro* mutants are so attenuated that these mutants are avirulent even for a sensitive model such as gnotobiotic pigs (33). The extreme attenuation was probably a reason why in at least some cases the *aro* mutants were not immunogenic and did not efficiently protect animals from subsequent infection, especially when highly virulent *Salmonella* strains were used for the challenge (17, 20). Despite this, inactivation of *aro* genes is one of the most frequently used methods for *S. enterica* attenuation.

The reduced virulence of *aro* mutants has been explained by their inability to produce aromatic metabolites, mainly aromatic amino acids such as phenylalanine, tyrosine, and tryptophan. Since amino acids are not freely available inside a host, *aro* mutants were expected to be incapable of intracellular replication. This has been indirectly supported by in vitro experiments in minimal media in which *aro* mutants did not grow as long as the aromatic amino acids or their precursors, *p*-amino benzoic acid or 2,3-dihydroxy benzoic acid, were added. However, if this was true, there would be principally no reason why mutants in biosynthetic pathways leading to the synthesis of other amino acids could be attenuated as well, something that has never been described and reported, at least as extensively as for the *aro* mutants.

Instead, *aro* mutants were occasionally described as having other defects. Although we did not investigate this in a greater detail during our previous study on respiration deficient mutants, we noticed that *aro* mutants stained less efficiently with rhodamine and therefore seemed to be defective in respiration (23). A similar hypothesis has also been proposed for the *aro* mutants in *Listeria* spp. (30). *aro* mutants were also described as having defects in motility (1). The attenuation of serovar Typhimurium *aro* mutants for gnotobiotic pigs free of any other bacteria also indicated a pleiotropic effect of *aro* mutation since, at least inside the gut, the *aro* mutants of serovar Typhimurium should not suffer from a lack of nutrients in the absence of any other competitive microflora. Despite this, the *aroA* mutant was attenuated and even did not trigger an innate immune response and cytokine production in gnotobiotic pigs (33). This finding suggested that *aro* mutants not only cannot replicate within a host due to the inability to synthesize aromatic amino acids but may also be defective in cytoplasmic or outer membrane or periplasmic space function, which could make them more sensitive to some components of the innate immune response.

This was a reason why we looked in a greater detail at the properties of *aro* mutants. To avoid association with a particular strain or serovar, we assessed the ability of *aroA* and *aroD* mutants in serovar Enteritidis and serovar Typhimurium and found that the *aro* mutants were highly sensitive to complement killing, EDTA, ovotransferrin, and the action of albumen.

MATERIALS AND METHODS

Bacterial strains and growth media. Serovar Enteritidis 147 used in the present study is a phage-type PT4 poultry isolate (19) with a high level of virulence (an oral 50% lethal dose for mice of 10^2 CFU). As a representative of serovar Typhimurium, the LT2 strain was selected. *aroA*, *aroC*, *aroD*, and *rfaC* mutants were generated by one-step λred recombination of PCR products (6). The primers used for the amplification of pKD46 plasmid with 44-bp overhangs specific to *aroA*, *aroC*, *aroD*, and *rfaC* are listed in Table 1. After the generation of primary mutants (except for the *rfaC* mutant), the *aroA*, *aroC*, and *aroD* mutations were transduced by P22 phage into a fresh wild-type strain. The transduction was not possible in the *rfaC* mutant due to its incomplete O antigen, resulting in resistance to P22 phage infection. The *rfaC* mutant was included as

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TABLE 1. Primers used in this study

Primer[a]	Sequence[b]
aroA44F	ATGGAATCCCTGACGTTACAACCCATCG CGCGGGTCGATGGCGC GTGTAGGCTGGAGCTGCTTC
aroA44R	TTAGGCAGGCGTACTCATTCGCGCCAGT TGTTCGAAATAATCAG CATATGAATATCCTCCTTAG
aroD44F	TTACGCCTGGTGCAATATAGTTAATACG GTACGCAGATCGGCTA GTGTAGGCTGGAGCTGCTTC
aroD44R	ATGAAAACCGTAACTGTAAGAGATCTC GTGGTTGGCGAAGGCGC CATATGAATATCCTCCTTAG
aroC44F	TTACCAGCGTGGAATCTCTGTCTTTACA TCCGCATTCTGTGCCC GTGTAGGCTGGAGCTGCTTC
aroC44R	ATGGCAGGAAACACAATTGGACAACTC TTTCGCGTAACCACTTT CATATGAATATCCTCCTTAG
aroAFor	ACGGTTAATCCGGAAGATTC
aroARev	TACATCCTGCCAGTAGCGTG
aroDFor	CACCGAATGTGTTTATAATC
aroDRev	TTACTCCACTATTATCCCTG
murAFor	TCAGAGTGTGCTGATGAATG
murARev	GCGCACGCATGAGTTTATCG

[a] Primers designated with a "44" were used in PCRs prior to the λred recombination; the remaining primer pairs were used for cloning the *aroA*, *aroD*, and *murA* genes.

[b] Primers for the λred recombination were 64mers, and 44-nucleotide sequence gene-specific extensions are shown on separate lines from the 20-nucleotide sequence allowing amplification with pKD3 as a template.

a control since the rough mutants are known to be sensitive to extracellular stresses (2, 25, 29). The presence of gene cassettes interrupting the *aro* genes was confirmed phenotypically by newly acquired antibiotic resistance and the inability of mutants to growth in minimal medium and genotypically by PCR with a primer pair specific for the insert-flanking DNA junction. All of the transductants were also confirmed to be sensitive to P22 phage used for the transductions.

For complementation, *aroA*, *aroD*, and *murA* were amplified by PCR using a PCR Master Mix kit from Qiagen. The genes were amplified, including their natural promoters, and cloned into pCR2.1 by using the TOPO cloning system (pCR TOPO cloning kit; Invitrogen) according to the instructions of the manufacturer. The gene *aroA* was cloned together with the *serC* gene located upstream because these genes form an operon with a single promoter upstream of the *serC* gene (7, 8, 15). Since the promoter of *murA* has not been experimentally determined, the forward primer was designed 291 bp upstream of the *murA* start codon to avoid cloning the intact *yrbA* gene located upstream. After the selection of pAroA, pAroD, and pMurA plasmids in *Escherichia coli* by PCR using a forward primer from the plasmid sequence and a reverse primer from the particular gene sequence, the plasmids were purified by using a QIAprep spin miniprep kit from Qiagen and electroporated into the appropriate *Salmonella* strain.

Resistance to selected compounds with antimicrobial activity. Resistance of *aro* mutants and other strains was tested against normal and heat-inactivated (30 min, 56°C) porcine serum, EDTA, albumen, and bile salts. In addition, antimicrobial peptides, including polymyxin, nisin, azurocidin, indolicidin, cecropin, and individual components of albumen with known antibacterial activity, such as ovotransferrin, cystatin, trypsin, avidin, lysozyme, and ovalbumin, were also tested. All of these compounds were obtained from Sigma, and either as liquid or dissolved in water were mixed with an equal volume of LB broth and then serially diluted in LB broth with a twofold dilution step in a 96-well microplate. Each well of the microplate was then inoculated with a strain of interest and, after 24 h of incubation at 37°C, the MIC was visually determined. For strains containing recombinant plasmids with *murA*, ampicillin was also added to the microplates to restrict the growth of bacteria that would eventually eliminate the recombinant plasmid.

Gene expression in the *aroD* mutant. The genomewide transcriptional activity of the *aroD* mutant in serovar Typhimurium LT2 strain was assessed by using microarray analysis. Total RNA was purified from the wild-type serovar Typhimurium LT2 and its isogenic *aroD* mutant grown for 18 h in 20 ml of LB medium at 37°C. After centrifugation, the whole culture was used for total RNA purification with an RNeasy minikit from Qiagen. Approximately 5 μg of total RNA was reverse transcribed and labeled with Cy3- or Cy5-CTP by using a LabelStar Array kit from Qiagen.

Microarray chips were prepared by spotting 5′-amino-linker-modified 70mer oligonucleotides covering both the serovar Typhimurium and the serovar Typhi genomes (*Salmonella* Genus AROS V1.0; Operon, Cologne, Germany). The oligonucleotides were resuspended in MicroSpotting Solution Plus (Telechem International, Inc., Sunnyvale, CA) buffer and spotted onto Nexterion Slide E epoxysilane-coated substrate microarray glasses (Nexterion, Jena, Germany). Hybridization and posthybridization washes were performed by using a Nexterion slide 70mer kit exactly according to the instructions of the manufacturer. After the hybridization, the microarray slides were dried and subjected to scanning with the ScanArray from Perkin-Elmer.

The microarray analyses have been repeated three times, always in a dye-swap experimental setup. The raw datasets were processed as follows. Only spots flagged as being of good quality were considered for the analysis, and $\log_2$ ratio values were averaged between dye-swap experiments, resulting in three preprocessed datasets. From all of the spots present on the microarray chip, 2,743 were of good quality signal in all three datasets, and these were used for the analysis. The search for differentially expressed genes was performed by significance analysis of microarrays (34) using the Excel version with the FDR value set to 0.05. Raw data from the microarray analysis were deposited in the GEO database under accession number GSE9411.

TABLE 2. Survival of *aroA*, *aroD*, and *rfaC* mutants in porcine blood serum

Strain	MIC (%) ± SD[a]		
	No plasmid	pAroA	pAroD
SE147	50 ± 0*	ND	ND
SE147 (*aroA*::Cm)	3.61 ± 0.93	50 ± 0*	2.88 ± 1.19
SE147 (*aroD*::Cm)	3.12 ± 1.56	3.94 ± 4.26	50 ± 0*
SE147 (*rfaC*::Cm)	10.70 ± 3.41	9.38 ± 3.32	12.5 ± 0
STM LT2	50 ± 0*	ND	ND
STM LT2 (*aroD*::Cm)	5.26 ± 2.19	ND	50 ± 0*

[a] The MICs of porcine serum in LB medium for the strains with or without the pAro plasmids are shown. ND, not done. *, Mixing equal volumes of LB and porcine serum did not affect the growth of the *Salmonella* at all.

RESULTS

Sensitivity of *aro* mutants to porcine serum. Although *Salmonella* is predominantly an intracellular parasite, it can occasionally be found extracellularly exposed to the complement present in blood serum (4). This was the reason we have tested its resistance to blood serum. *aroA* and *aroD* mutants in serovar Enteritidis were unable to survive in porcine blood serum. The serum had to be diluted more than 10 times to allow *aro* mutants to grow. Since the mutants were highly sensitive to normal but not to the heat-inactivated serum (results not shown), we concluded that the mutants were sensitive to complement killing. The *aroD* mutant in serovar Typhimurium behaved essentially in the same way as the *aro* mutants in serovar Enteritidis. Resistance to complement killing was dependent also on full-sized O antigen since the *rfaC* mutant was approximately five times more sensitive to complement killing than was the wild-type strain. However, in the *aro* mutants the wild-type phenotype could only be restored by transformation with the appropriate pAroA or pAroD plasmid (Table 2).

Sensitivity of *aro* mutants to albumen. Since the most frequent mode of serovar Enteritidis transfer to the human population is through eggs and egg products, we were interested in the survival of *aro* mutants in the presence of albumen. In this

TABLE 3. Resistance to antimicrobial action of albumen

Strain	MIC (%) ± SD[a]		
	No plasmid	pAroA	pAroD
SE147	43.75 ± 15.31	ND	ND
SE147 (*aroA*::Cm)	1.82 ± 0.59	18.75 ± 8.84	1.56 ± 0
SE147 (*aroD*::Cm)	2.08 ± 0.74	6.77 ± 4.48	37.5 ± 12.5
SE147 (*rfaC*::Cm)	19.2 ± 14.79	14.58 ± 7.8	14.58 ± 7.8
STM LT2	20.63 ± 8.75	ND	ND
STM LT2 (*aroD*::Cm)	2.5 ± 1.88	ND	37.5 ± 12.5

[a] The MICs of albumen (as a percentage) in LB medium are shown. ND, not done.

assay, both the *aroA* and the *aroD* mutants of serovar Enteritidis were >20 times more sensitive to the action of albumen than was the wild-type strain. The *aroD* mutant in serovar Typhimurium behaved essentially in the same way as the *aro* mutants in serovar Enteritidis. Compared to the wild-type strains, the *rfaC* mutant required only one additional twofold dilution of albumen in LB medium for unrestricted growth and was therefore more resistant to albumen than were the *aro* mutants. As in the case of the blood serum, complementation with the appropriate pAroA or pAroD plasmid restored the wild-type level of resistance in the *aro* mutants (Table 3).

Sensitivity of *aro* mutants to EDTA. Increased sensitivity of the *aro* mutants to serum and albumen killing indicated a defect in the outer membrane and/or periplasm structure. EDTA is known to affect these structures, and we therefore determined the resistance of the *aro* mutants to EDTA. Both the *aroA* and the *aroD* mutants were highly sensitive to the presence of EDTA. The mutants were >50 times more sensitive than was the wild-type strain and, since the MIC for EDTA for the wild-type strains was ~6 mM, *aro* mutants grew only when EDTA was diluted to less than 10 μM. The *aroD* mutant in serovar Typhimurium behaved essentially in the same way as the *aro* mutants in serovar Enteritidis. As in the case of the blood serum or albumen sensitivity, complementation with appropriate the pAroA or pAroD plasmid restored the wild-type level of resistance in the *aro* mutants (Table 4).

Sensitivity of *aro* mutants to other compounds with antimicrobial activities. *aroA* and *aroD* mutants were as resistant as the wild-type strains to the action of bile salts (MIC = 5%) and polymyxin (MIC = 2.5 μg/ml). Cystatin at 100 μg/ml of LB broth, trypsin (20 mg/ml), avidin (1 mg/ml), lysozyme (100 μg/ml), ovalbumin (20 mg/ml), nisin (2.5 mg/ml), azurocidin (10 μg/ml), indolicidin (20 μg/ml), and cecropin (20 μg/ml) did not suppress the growth of either the wild-type strain or the

TABLE 4. Resistance to antimicrobial action of EDTA

Strain	MIC (nM) ± SD[a]		
	No plasmid	pAroA	pAroD
SE147	6.42 ± 4.08	ND	ND
SE147 (*aroA*::Cm)	0.11 ± 0.05	5.73 ± 3.33	0.11 ± 0.04
SE147 (*aroD*::Cm)	0.09 ± 0.04	1.39 ± 1.13	5.8 ± 3.09
SE147 (*rfaC*::Cm)	0.22 ± 0.11	0.23 ± 0.07	0.22 ± 0.13
STM LT2	8.04 ± 2.82	ND	ND
STM LT2 (*aroD*::Cm)	0.14 ± 0.1	ND	4.17 ± 1.47

[a] The MICs of EDTA in LB medium are shown. ND, not done.

TABLE 5. Genes up- or downregulated in serovar Typhimurium *aroD* mutant grown for 24 h in LB broth

Category and gene code	Gene name	Log_2 fold induction
Upregulated in *aroD*		
STM1583		3.83
STM1172	*flgM*	3.71
STM4030		3.56
STM1183	*flgK*	3.45
STM3307	*murA*	3.36
STM3670		3.26
Downregulated in *aroD*		Log_2 fold suppression
PSLT014	*orf6*	7.96
PSLT093		4.50
PSLT069	*psiB*	3.47
PSLT097	*traF*	3.37
PSLT064		3.23
PSLT099	*trbB*	3.09
PSLT005	*tap*	3.03
PSLT106		2.97
STM0159		8.03
STM1018		5.28
STM1838	*yobF*	4.45
STM0831	*dps*	4.04
STM1012		3.68
STM2796	*yqaE*	3.10
STM3656	*glyQ*	3.06

aroA and *aroD* mutants and, since at least some of the concentrations tested were already quite high and thus biologically irrelevant, we did not attempt to determine the actual MICs. Ovotransferrin was the only compound to have a different effect on the wild-type strain and the *aroA* and *aroD* mutants. Although the wild-type serovar Enteritidis grew at ovotransferrin concentration of 20 mg/ml, the MIC for the *aroA* and *aroD* mutants was 0.625 mg/ml, and this defect could be restored by the appropriate pAroA or pAroD plasmid. The MIC of ovotransferrin for the control *rfaC* mutant was 2.5 mg/ml.

Microarray analysis. Microarray analysis revealed 21 genes that were differentially expressed in serovar Typhimurium LT2 and its isogenic *aroD* mutant. Fifteen genes were downregulated in *aroD* mutant, and six were upregulated.

Among the genes downregulated in the *aroD* mutant, eight were localized on the virulence plasmid. Of the remaining suppressed genes, only two have an assigned function. *glyQ* encodes glycine tRNA synthetase, and *dps* codes for stress-induced DNA binding a protecting protein (Table 5).

Within the upregulated genes, two genes were related to flagellum expression regulation. *flgK* encodes the flagellum hook protein, and *flgM* encodes the antisigma factor negatively regulating flagellum expression. The most interesting finding, however, was the upregulation of *murA*, since MurA is directly involved in the synthesis of bacterial peptidoglycan (Table 5).

***murA* and sensitivity to antimicrobial agents.** MurA is the first protein catalyzing the synthesis of peptidoglycan. Its deletion is lethal to bacteria, while its upregulation is known to lead to increased resistance to fosfomycin (3, 16). To test the hypothesis that the phenotypes observed in *aro* mutants could be associated with the upregulation of *murA*, we cloned *murA* into a multicopy vector, generating plasmid pMurA, and we

TABLE 6. Resistance of pMurA transformed serovar Enteritidis to different agents with antimicrobial activities

Strain	MIC ± SD				
	Fosfomycin (mM)	Serum (%)	Albumen (%)	EDTA (mM)	Ovotransferrin (mg/ml)
SE147	2.08 ± 0.58	50 ± 0	43.75 ± 15.3	6.42 ± 4.08	>20
SE147 (*aroA*::Cm)	1.04 ± 0.29	3.61 ± 0.93	1.82 ± 0.59	0.11 ± 0.05	0.625 ± 0
SE147 (*aroD*::Cm)	1.04 ± 0.29	3.12 ± 1.56	2.08 ± 0.74	0.09 ± 0.04	0.625 ± 0
SE147(pMurA)	3.75 ± 1.58	50 ± 0	4.69 ± 1.56	0.26 ± 0.09	>20

[a] The MICs for fosfomycin, serum, albumen, EDTA, and ovotransferrin, all diluted in LB medium, are shown. The characteristics of the wild-type strain and *aro* mutants are included for comparison.

compared the fosfomycin resistance of the wild-type strain with or without pMurA to that of the *aroA* and *aroD* mutants. Furthermore, the wild-type strain transformed with the pMurA was also tested for its sensitivity to porcine serum, albumen, ovotransferrin, and EDTA.

Transformation of SE147 with pMurA resulted in an increase of its resistance to fosfomycin, a finding consistent with previous observations (16). Both *aroA* and *aroD* mutants were, on the other hand, more sensitive to the fosfomycin than was the wild-type strain (Table 6). The pMurA transformant was also sensitive to EDTA and albumen in range similar to that of the *aro* mutants. Unlike the *aro* mutants, the pMurA transformant was completely resistant to the action of porcine serum and ovotransferrin (Table 6).

PEP competition. MurA is structurally similar to AroA, both utilizing phosphoenolpyruvate (PEP) as a substrate (9). We therefore speculated that *Salmonella* attempted to resolve the reduced consumption of PEP in *aro* mutants by increased PEP consumption in the MurA pathway through the *murA* upregulation. If such a hypothesis was correct, then the *aroC* mutant, which catalyzes the synthesis of aromatic core one step downstream from the action of AroA, might display characteristics different from the *aroA* and *aroD* mutants. However, when the *aroC* mutant was tested for sensitivity to porcine serum, albumen, ovotranferrin, and EDTA, its behavior was identical to that of *aroA* and *aroD* mutants (data not shown). This suggests that either the hypothesis was not correct or the AroA-catalyzed step is under negative feedback control by the product which, when not utilized by AroC, leads to a decrease in AroA substrate consumption and PEP accumulation.

DISCUSSION

aro mutants are well known to be attenuated for different animals, including humans. This has been traditionally explained by the inability of these mutants to replicate within a host in which aromatic compounds are not freely available. Although this feature can contribute to the reduced virulence of *aro* mutants, these mutants may also be defective in motility or respiration, probably due to the inefficient production of ubi- and menaquinones (1, 30). Furthermore, when working with *aro* mutants in serovar Typhimurium in gnotobiotic pigs (33), we observed that the mutants were sensitive to complement killing (unpublished observations). In the present study we therefore focused on the properties of different *aro* mutants in detail.

aro mutants, regardless of the *S. enterica* serovar, were highly sensitive to complement killing, albumen, ovotransferrin, and EDTA, all indicating a cell envelope biosynthesis defect. Concerning the complement killing, human or chicken sera were tested as well with results identical to that of porcine serum (data not shown). In addition, the same phenotype was observed in several other serovar Enteritidis strains into which we transduced the *aro* mutations. We also tested completely independent transposon mutants from our previous studies (24, 33), and in all of the *aro* mutants we found the same phenotype (data not shown). This clearly demonstrates that the phenotype was tightly associated with the *aro* mutation and not with only a single strain or serovar.

Although in some cases the behavior of *aro* mutants was similar to the behavior of the rough *rfaC* mutant, the sensitivity of *aro* mutants was not due to the defect in O-antigen synthesis since all of the *aro* mutants, unlike the *rfaC* mutant, could be agglutinated with O-antigen-specific sera and were sensitive to P22 phage. Furthermore, unlike the *aro* mutants, the *rfaC* mutant was also defective in invasion of the cell culture (results not shown) and was less sensitive to serum, albumen, and ovotranferrin killing. The *rfaC* mutant was also highly sensitive to bile salts action, which both *aro* mutants survived, as did the wild-type strain. Due to the several similarities and differences between *rfaC* and *aro* mutants, it was obvious that the *aro* mutants were defective in some components of bacterial cell envelope different from the O antigen.

To gain a better insight into the gene expression of *aro* mutants, the *aroD* mutant of serovar Typhimurium grown in LB broth was subjected to microarray analysis. Eight of fifteen genes suppressed in the *aroD* mutant were encoded by the virulence plasmid; the meaning of this remains unclear. Another gene suppressed in the *aroD* mutant was *dps*, which codes for ferritin-like DNA binding and protecting protein (13). Its decreased transcription in the *aroD* mutant might be one of the reasons for *aro* mutant attenuation.

Although only six genes were found to be upregulated in the *aroD* mutant (the function of three of them had been previously determined), they clearly fit into the phenotypes of *aro* mutants. *flgM*, the flagellum antisigma factor, was one of these genes. Its upregulation may explain the previously described reduced flagellation of *aro* mutants (1) and might be caused by the upregulation of the *flhDC* master operon, which was shown to be affected by outer membrane integrity in *Yersinia enterocolitica* (21) and the expression of which, although not reaching a statistically significant threshold value, was ca. 30% higher in the *aroD* mutant than in the wild-type strain. However, this probably did not contribute to the *aro* mutant's attenuation since completely aflagellated *fliC* mutant was as virulent for mice as was the wild-type strain (unpublished observations).

The upregulation of *murA* could contribute to the cell envelope defects and the EDTA and albumen sensitivity of *aro* mutants since the substrate for MurA is UDP-*N*-acetylglucosamine. Interestingly, the same substrate is a starting point for the biosynthesis of lipid A and O antigen (11, 28). Increased consumption of UDP-*N*-acetylglucosamine in *aro* mutants may lead to its decreased availability for the synthesis of lipid A, resulting in decreased resistance to EDTA and albumen. Mere upregulation of *murA* in *aro* mutants, however, could not explain their sensitivity to porcine serum and ovotransferrin. We can exclude that the serum sensitivity of *aro* mutants could be associated with reduced production of flagella since the nonflagellated, nonmotile *fliC* mutant of serovar Enteritidis 147 was fully resistant to porcine serum (unpublished observations). Whether the suppression of *dps* in the *aroD* mutant or any of the genes of unknown functions, or even a particular combination of some of the misregulated genes in the *aroD* mutant, was responsible for its complement and ovotransferrin sensitivity thus remains unclear but is highly probable. The defect of the *aro* mutants could be also caused by the combined effect of *murA* upregulation and a lowered availability of aromatic compounds, either the aromatic amino acids or ubi- or menaquinones, as a direct effect of the *aro* mutations. Finally, it cannot be excluded that *murA* upregulation is a consequence of and not a reason for the defects observed in *aro* mutants. It cannot be ruled out that serovar Enteritidis attempts to solve the defect caused by *aro* mutations, e.g., an accumulation of polysaccharide intermediates by upregulation of *murA*. In such a case, the properties of the *aro* mutants need not overlap with the properties of the strain with upregulated *murA*. Though we do not know the exact reason for *aro* mutant defects, the fact that *aro* mutants are sensitive to chelating agents may explain their inability to survive for prolonged periods inside macrophages (18), which release Nramp1 protein with chelating properties into the *Salmonella*-containing phagosome (12).

We have shown that *aro* mutants are highly sensitive to the action of natural agents with antimicrobial activities. It is uncertain to what extent these features are relevant for the *aro* mutant's attenuation compared to its inability to synthesize aromatic amino acids, since mutants defective in cell wall or outer membrane functions, such as rough mutants, have been used for a long time as safe and effective live vaccines (26). Our results also indicated that *aro* mutants might be even safer than rough mutants due to their usually higher sensitivity to the antimicrobials tested. This is also supported by observations in gnotobiotic pigs in which rough mutants caused limited damage that led to an induction of an inflammatory immune response, whereas the *aroA* mutant was so attenuated that it even did not induce any proinflammatory cytokine response (27, 33).

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