INTRODUCTION

Foodborne Salmonella spp. continue to be a major cause of illnesses in US human populations with more than 1 million cases reported annually, hospitalizations being required for some individuals, and in some cases death occurring (Scallan et al., 2011; Howard et al., 2012). Poultry and chicken in particular continue to be identified as leading food sources for human salmonellosis (Mead et al., 2010; Finstad et al., 2012). Although the ranking shifts to some extent, the primary serovars consistently isolated from poultry include Salmonella enterica serovar Enteritidis along with Salmonella Kentucky, Salmonella Typhimurium, and Salmonella Heidelberg followed by several others at lower frequencies (Foley et al., 2011; Finstad et al., 2012). Of these serovars, Salmonella Kentucky has only limited linkage to human salmonellosis, whereas Salmonella Typhimurium is the serovar most often identified with reported cases (Foley et al., 2011; Finstad et al., 2012).

The ecology and occurrence of Salmonella serovars in poultry versus those directly associated with human salmonellosis remain difficult to quantify in part due to serovar variability in recovery from culture media (Mead et al., 2010). The complexity of the poultry environment-Salmonella relationship may also be a factor as well (Park et al., 2008). Although much is now generally known on virulence gene regulation and pathogenesis phenotype for particular Salmonella serovars and their interaction with hosts as a pathogen, less is known about their physiological and metabolic responses to the environment they encounter during the poultry production cycle (Marcus et al., 2000; Park et al., 2008; Dunkley et al., 2009a; Malik-Kale et al., 2011; Calenge and Beaumont, 2012). During production and processing, poultry exposure to Salmonella can originate from a wide variety of sources ranging anywhere from feed, various biological vectors, and even aerosols (Murray, 2000; Pillai and Ricke, 2002; Maciorowski et...
al., 2004; Park et al., 2008). Each of these sources and their corresponding environments represents potential combinations of unique challenges to Salmonella spp. and their ability to counter with the appropriate stress response(s). This in turn can cause great difficulty in designing effective intervention measures at different stages of poultry production.

A classic example of this complexity is the response of Salmonella to lowering of pH either via the generation of organic acids by fermentative bacteria in the avian gastrointestinal tract (GIT) or as an intervention added to feed or potentially during processing (Ricke, 2003b; Mead et al., 2010). At first glance this would seem to be a fairly straightforward single stress response, but as more characterization has been done, it is now known to be much more complicated. Salmonella are capable of becoming resistant to lower pH levels via the classic acid tolerance mechanisms that are well established for several bacteria (Foster, 1999). However, exposure to high concentrations of organic acids at neutral pH that are typically generated by GIT bacteria can also stimulate acid tolerance, modulate virulence gene response, and cause cross protection to unrelated antimicrobials (Kwon and Ricke, 1998; Durant et al., 2000; Kwon et al., 2000). This may in part be due to the fact that Salmonella can grow anaerobically and generate some of these same fermentation acids at concentrations similar to the GIT tract microflora (Dunkley et al., 2009b). In addition, there are subtle strain and serovar differences in acid tolerance and virulence gene expression which may explain some of the variation seen in frequency of certain serovars in poultry (Joerger et al., 2009, 2012; González-Gil et al., 2012; Shah et al., 2012).

The other factor that complicates assessment and prediction of Salmonella prevalence in poultry is the intermingling of virulence and metabolic responses during interaction of the pathogen with the host. Rohmer et al. (2011) has hypothesized that much of the genetic evolution of microorganisms to become pathogenic has been due to a selection toward better acquisition of nutrients available in the corresponding host tissues. The enhancement of virulence gene expression and subsequent systemic tissue invasion that occurs during Salmonella Enteritidis infection of laying hens undergoing feed removal and subsequent nutrient deprivation would certainly support this concept (Durant et al., 1999; Ricke, 2003a; Dunkley et al., 2007). Likewise, the ability of Salmonella Typhimurium to induce inflammation by utilizing tetrathionate respiration to outcompete GIT microflora represents a combination of pathogenesis and energy metabolism to manipulate the GIT environment to its advantage (Winter et al., 2010; Rohmer et al., 2011). Such results suggest that changes in Salmonella metabolism due to environmental changes may either initiate pathogenesis as occurs in Salmonella Enteritidis or, as in the case of Salmonella Typhimurium, pathogenesis is first used to create a metabolic competitive edge over other microorganisms.

Consequently, it is quite possible that both virulence and metabolic genes of pathogens such as Salmonella are much more codependent than previously thought. This may explain why it remains difficult to achieve consistent widespread control of Salmonella spp. in poultry with the use of single intervention measures and in turn why multiple interventions tend to be more effective. Now that entire Salmonella genomes have been sequenced (McClelland et al., 2001; Parkhill et al., 2001), there should be much more opportunity to identify optimal targets for multiple antimicrobial/intervention steps. However, even with the advent of advanced sequence technology and establishment of entire Salmonella genomes there remains a considerable gap between initial identification of genes and the actual phenotypic roles they may play in the environment. Therefore, identifying regulatory networks and quantitating gene expression levels under varied environmental conditions are still needed to establish functionality. This involves transcriptome-based tools such as microarrays that enable comprehensive genomic screening and direct quantitative assessment of gene expression levels. The goal of this review is to discuss application of microarrays for identification of Salmonella in poultry environments as well as transcriptome responses to stressors likely to be encountered in poultry environments.

**BASIC CONCEPTS IN MICROARRAY ANALYSES**

Complete genome sequences have added considerably to the database for many of the foodborne pathogens of primary concern and offer tremendous insights to the organism’s entire genomic capacity. However, to fully explore the function of each gene requires profiling of gene expression of the microorganism during exposure to either treatment or environmental conditions of interest. The availability of whole genome sequences provides a catalog of all genes of an organism, and this information has led to the construction of DNA microarrays (Lucchini et al., 2001). In DNA microarray technology, functionality is detected when a gene is transcribed in response to an external signal that requires a gene to function (Cummings and Relman, 2000). Gene expression profiling or transcriptome analysis on a genomic scale is accomplished with glass microarray slides, which are constructed by cross-linking either oligonucleotides (referred to as oligonucleotide microarrays) or PCR products representing individual genes (referred to as cDNA microarrays on the glass surface of the array; Lucchini et al., 2001; Sirsat et al., 2010). Consequently, a DNA microarray is simply a collection of microscopic DNA spots attached to a solid surface (Sirsat et al., 2010).

The core principle behind a microarray assay is hybridization between 2 DNA strands. Once the sequence-specific nucleic probes are attached to the mi-
Microarray surface, they serve as hybridization probes for pools of labeled target nucleic sequences where the amount of label detected represents the abundance of the corresponding target sequence at a specific probe site (Douglas et al., 2013). A high number of complementary base pairs in a nucleotide sequence means tighter noncovalent bonding between the 2 strands. After washing off of nonspecific bonding sequences, only strongly paired strands will remain hybridized. Therefore, fluorescently labeled target sequences that bind to a probe sequence generate a signal that ultimately depends on the strength of the hybridization determined by the number of paired bases and the hybridization conditions. Microarrays use relative quantification in which the intensity of the hybridization in question is compared with the intensity of the same hybridization under a different condition, and the identity of the hybridization is known by its position. These changes in DNA-based microarray transcriptome responses have a wide variety of potential applications for developing a better understanding of Salmonella spp. capabilities and their ability to persist as pathogens. For example, application of microarrays has helped to identify new Salmonella genomic regulatory sites for controlling virulence gene expression and also provided evidence for a more highly integrated genome-wide response and subsequent adaptation after exposure to antibiotics (De Keersmaecker et al., 2005; Dowd et al., 2007). In the following sections, microarray studies comparing Salmonella gene expression levels under various experimental treatments that simulate poultry production and processing conditions as well as applicable Salmonella comparative genomics are discussed.

**Salmonella Microarrays and Comparative Genomics**

The high-throughput capacity of DNA microarrays offers tremendous utility for identification and genotyping of Salmonella for comparative genomics (Gold-schmidt, 2006; Sirsat et al., 2010). By using one genome as a reference and spotting on the microarray slide the second genome to be compared, the resulting extent of hybridization between the 2 sequences can be used to determine the presence or absence of similar sequences in the 2 genomes (Douglas et al., 2013). This has tremendous potential for precisely identifying minor differences in genomic DNA that can help not only with differentiating Salmonella strains but also allows for better targets for developing highly specific molecular probes for traceability of multiple closely related strains. For example, Chan et al. (2003) using a Salmonella Typhimurium DNA microarray to conduct comparative hybridizations with serovars and strains of Salmonella enterica (subspecies I and IIIA) and Salmonella bongieri observed agreement with previously published multilocus enzyme multilocus data. Although this confirmed genetic difference was reflected by the serogroupings, they noted variability in pathogenicity genes among serovars that aligned with their corresponding host specificity that could not be accounted for by serogrouping alone. More recently, Huehn et al. (2010) expanded this comparison to more than 500 Salmonella enterica ssp. enterica strains originating from a wide range of sources both geographically as well as various hosts. After using PCR to prescreen for 10 virulence encoding genes that were representative of either highly conserved (genomic pathogenicity islands) or variable genomic regions (prophages and plasmids), they concluded that only 14 virulotypes could be distinguished and these did not substantially vary among host source or geographical location. Consequently, a representative 77 member subset of strains was compared using specifically constructed microarrays containing 102 virulence and 49 resistance determinants. Although generally paralleling the PCR profiles, variability of virulence genes encoded on prophages, plasmids, or in the fimbrial clusters did occur. Huehn et al. (2010) concluded that these results were not necessarily surprising, particularly the fimbriae clustering outcome, because fimbriae are involved in initial attachment to host cells and are considered host specific.

This ability to more precisely detect genomic differences has potential to serve as a highly effective tool for conducting Salmonella traceability studies in samples originating from complex matrices such as poultry feeds where knowing contamination sources could be an important control measure (Jarquin et al., 2009). The possibility of this was suggested in studies conducted by Alvarez et al. (2003) in which they initially identified a Salmonella enterica serovar California strain occurring in Spanish feed mills using conventional genotyping methods. They conducted DNA microarray hybridization with Salmonella Typhimurium to establish that several gene clusters were missing from the California strain including one of the fimbrial operons and all 4 active prophages. More recently, Koyuncu et al. (2011) used a commercially available Salmonella DNA microarray platform that consisted of both a combination of genes that exhibited no sequence variation among serotypes along with serotype-specific gene probes to detect animal feed components artificially contaminated with Salmonella. Based on comparisons with traditional cultural methods, they concluded that a selective enrichment step was required to sufficiently remove background interference to achieve successful identification by the DNA microarrays. This is not a surprising result because similar conclusions have been drawn from PCR studies involving Salmonella identification and quantitation in poultry feeds (Maciorowski et al., 2000, 2005; Schultz et al., 2012). Further refinements in choice of enrichment media and potentially the use of Salmonella-specific immunomagnetic beads for initial recovery of Salmonella in these types of matrices may also be warranted to improve accuracy (Dunkley et al., 2007; Park et al., 2011; Schultz et al., 2012).
MICROARRAY ANALYSES OF SALMONELLA AND SUBLETHAL HEAT EXPOSURE

Sirsat et al. (2011a) used a Salmonella Typhimurium oligonucleotide microarray consisting of 43- to 45-mer sequences of 1,152 genes from virulence, membrane, stress, quorum sensing, and transcriptional regulation functional regions to determine the influence of sublethal heat stress (42°C) on virulence gene expression. Growth was not altered when bacterial cells were grown at the control temperature (30°C) versus the elevated temperature. This tolerance appeared to be reflected in the upregulation of stress genes (rpoS, rpoH, and rpoE of the RpoS regulon) that confers resistance to stationary cells exposed to a range of environmental stresses including in addition to heat, acids, osmotic shock, and starvation (Loewen and Hengge-Aronis, 1994; Sirsat et al., 2011a). When gene responses associated with virulence were compared between the 2 temperatures, sublethal heat induced Salmonella Pathogenicity Island-2 (SPI-2) genes (ssaJ, ssaP, sseA, and sseB) required for intracellular replication in host cells after invasion but repressed SPI-1 genes responsible for invasion of host epithelial cells (Marcus et al., 2000). However, increasing temperature did induce fimbiae genes (stbA, safC, and safD) associated with adherence to intestinal cells (McClelland et al., 2001). Although the transcriptional virulence responses to sublethal heat of increasing adherence gene levels but actually repressing invasion gene responses would appear contradictory, this relationship was in fact supported by the almost 2-fold increase in Salmonella Typhimurium adhesion to Caco-2 cells versus no change in percentage bacterial invasion (Sirsat et al., 2011a).

Taken together, these responses suggest that Salmonella exposed to even subtle shifts in temperature may alter its pathogenesis phenotype fairly dramatically. Consequently, this has potential colonization implications in the poultry GIT where Salmonella encounters the 42°C body temperature of the bird. Likewise, the question remains on whether temperature shifts that occur during poultry processing increase nonspecific binding of foodborne Salmonella to surfaces such as chicken skin. This question could be partially answered by directly assessing transcriptional responses of Salmonella attached to chicken carcass surfaces. However, Sirsat et al. (2011b) determined that at least 8 log cfu/g of Salmonella cells would be required to ensure sufficient amounts of RNA are recovered for microarray analyses.

MICROARRAY ANALYSES OF SALMONELLA AND HEAT-ORGANIC ACID COMBINATIONS

Microarray expression analyses may have utility for poultry processing applications as well. Continuous application of certain antimicrobial compounds in poultry processing environments has the potential to generate a buildup of microbial resistance over time (Ricke et al., 2005). Simultaneous application of different antimicrobial chemical combinations or combining chemical and physical treatments as “multiple hurdle” interventions has been suggested as a means to achieve synergistic antimicrobial interactions and prevent pathogens from adapting and developing tolerance to certain antimicrobials (Leistner, 1992; Ricke et al., 2005). However, such synergism is not always a certainty, and in some cases the opposite may occur where cross-protection among antimicrobials generates subpopulations of microorganisms that become resistant to several antimicrobials because the microorganism carries genetic elements that trigger resistance to multiple stressors. For example, Kwon et al. (2000) demonstrated that Salmonella Typhimurium cells adapted to SCFA at neutral pH could also become more resistant to high pH, high osmolarity, and reactive oxygen.

Sirsat et al. (2010) suggested that screening genomic responses would be an efficient approach to identify combinations of antimicrobials where the individual antimicrobials are sufficiently distinct mechanistically to avoid initiating shared resistant genes. This approach has been demonstrated with Salmonella. Using raw chicken media, Milillo and Ricke (2010) reported that exposing Salmonella Typhimurium to a mild thermal treatment (55°C) combined with 2.5% organic acids for 1 min dramatically reduced viability versus virtually no reduction when treatments were applied singly.

In a follow-up study, Milillo et al. (2011) demonstrated that combinations of organic acids at pH 4 and mild heat (55°C) caused substantial changes in membrane permeability with the more lipophilic organic acids sodium lactate and sodium propionate versus sodium acetate, achieving synergistic reduction of viable cells under these specific conditions. When microarray analysis was conducted, gene responses to sodium acetate and sodium propionate were similar with minimal influence on virulence genes and stress genes but repression of several of the heat shock genes also occurred. They concluded that maintenance of membrane integrity was critical for Salmonella to remain viable and interventions that cause immediate membrane destabilization can lead to cell death from other factors present such as changes in intracellular pH. Although previous reports have indicated that organic acids can directly cause Salmonella virulence gene response, it may be simply a matter of exposure time (1 min in these studies versus 1 to 4 h in Durant et al., 2000) and the limited selection of virulence genes (hla and mviF gene fusions versus entire genome) examined by Durant et al. (2000). Damage to cells and resulting lethality are probably a function of exposure time and how the interventions are combined (simultaneously or sequentially). As more microarray studies are conducted, factors such as incubation conditions and the resulting microbial physiol-
ogy will need to be more precisely defined to sort out which factors have the most impact on transcriptional responses.

MICROARRAY ANALYSES OF SALMONELLA AND MAILLARD PRODUCTS

The chemical nature of the feed and food matrices with which Salmonella spp. are associated may also play a role in its growth and metabolism. When heat is applied to foods or feeds, nonenzymatic reactions occur between carbonyl substrates and free amino groups resulting in condensation of reducing sugars with amino groups, followed by the Amadori rearrangement and decomposition of ketose-containing compounds to advanced glycation end products, which further react to form an insoluble pigment, melanoidin (Hurrell and Finot, 1983; Friedman, 1996; Henle, 2005). These compounds are collectively referred to as Maillard reaction products (MRP) and their formation, particularly with essential dietary amino acids such as lysine, can result in reduced nutritional quality in foods and feeds due to the decrease in lysine availability (Carpenter and Booth, 1973; Friedman, 1996). However, these compounds can also influence microbial activity and, depending on the organism, can elicit antimicrobial properties against foodborne pathogens including Salmonella (Einarsson et al., 1983).

Previous work with Salmonella Typhimurium liquid growth cultures indicated that lysine-based MRP could influence expression of selected virulence genes in Salmonella Typhimurium, but in-depth analysis of metabolic genes had not been done (Kundinger et al., 2008). Chalova et al. (2012) compared transcriptome responses of Salmonella Typhimurium when grown in the presence of glucose-lysine-based MRP generated under low water activity (0.44) conditions that would be considered similar to those occurring during food or feed processing. When MRP were provided as the sole carbon source, bacterial growth response was biphasic with the MRP glucose consumed initially followed by disappearance of the remainder of the respective MRP. This was clearly reflected in the marked differences in energy metabolism-associated gene expression levels between cells grown on glucose versus those grown on MRP. In particular, MRP activated genes that are part of the glyoxylate carbon pathway employed by Salmonella under carbon limiting conditions. These limiting conditions were also indicated by the over 15-fold increases in starvation-induced genes encoding a starvation resistance regulatory protein (dps) and the carbon starvation regulatory protein (csA). Although it appears that MRP may force Salmonella into an energy scavenging mode, it is not clear how this may influence the response of Salmonella to other stressors such as the administration of antimicrobials used either in preharvest or postharvest poultry production.

CONCLUSIONS

Foodborne Salmonella continues to be problematic in poultry production. Part of this is due to the continuing emergence of new strains that represent subtle genetic alterations from previous isolates. High throughput genomic sequencing has now made it possible to detect these subtle genetic alterations and conduct comparative genetic comparisons among particular strains (Parkhill et al., 2001; McClelland et al., 2001; Holt and Jones, 2008; Reynolds et al., 2011). However, the patterns of Salmonella strains cycling through poultry production still remain somewhat unpredictable due to a limited knowledge of the relationship between Salmonella pathogenesis and establishment in poultry production environments. Although sequencing has added tremendously to the fundamental understanding of the Salmonella genome, this only represents an initial step in developing an in-depth assessment of the complex interactions that occur between the host and the pathogen. Understanding this interaction requires elucidating functionality not only for the known genes but also for the open reading frames in the genome where functionality has not been identified. Assigning functionality to open reading frame sequences on the Salmonella genome requires the ability to assess gene expression when Salmonella is exposed to the environmental condition of interest.

Generation of complete Salmonella genome sequences has led to the development of microarrays (Porwollik et al., 2002; Chan et al., 2003) that have enabled quantitative assessment of transcriptional responses. Microarrays represent a much more comprehensive strategy for studying Salmonella gene functions because the sequences spotted on the microarray slide can span the entire genome and gene expression is measured as the level of hybridization occurring between these DNA spots and the target nucleic acid. The resulting transcriptome profiles represent a genome-wide response of Salmonella to a specified environmental or treatment condition and are readily applicable to explore complex genetic mechanisms of this pathogen to survive and persist in poultry production. As discussed in this review, initial application of Salmonella microarrays have uncovered the complexity of the organism’s response to combining thermal with acid intervention steps as well as utilization of alternative carbon pathways when it grows on MRP. It is anticipated that further use of microarrays will provide key insights on how this organism can adapt and respond metabolically to multiple external stimuli typically present in poultry production systems.

Despite successful applications to a variety of research questions, there are limits to current microarray technology including differences in probe hybridization properties in individual experiments as well as replication difficulties across independent experiments (Marioni et al., 2008; Wang et al., 2009). In addition, microarray technologies are reliant on known genome sequences spotted on the microarray slide can span the entire genome and gene expression is measured as the level of hybridization occurring between these DNA spots and the target nucleic acid. The resulting transcriptome profiles represent a genome-wide response of Salmonella to a specified environmental or treatment condition and are readily applicable to explore complex genetic mechanisms of this pathogen to survive and persist in poultry production. As discussed in this review, initial application of Salmonella microarrays have uncovered the complexity of the organism’s response to combining thermal with acid intervention steps as well as utilization of alternative carbon pathways when it grows on MRP. It is anticipated that further use of microarrays will provide key insights on how this organism can adapt and respond metabolically to multiple external stimuli typically present in poultry production systems.
sequences precluding the ability to identify unknown or novel transcripts, or both (Marioni et al., 2008). Further advancement of high-throughput sequencing technologies has led to methods that can generate the sequence of the entire transcriptome (RNA sequencing or RNA-Seq), thus enabling discovery of novel transcripts and uncovering sequence variations (Wang et al., 2009; Oszolak and Milos, 2011; Raz et al., 2011; Mutz et al., 2013). In early work that demonstrated the improved utility of sequencing approaches for transcriptome assessment of Salmonella, Perkins et al. (2009) isolated RNA from Salmonella Typhi grown to mid-logarithmic growth phase, reverse transcribed the RNA to cDNA, and subsequently sequenced the DNA. Transcriptome sequence analysis revealed numerous transcribed regions contained in prophage and pseudogene locations as well as novel small noncoding RNA. As pointed out by Perkins et al. (2009) identifying these regions in the transcriptome offered better resolution between active and inactive transcription sites and the potential role these sites played in functions such as maintenance of the prophage. More recently, Kröger et al. (2012) used a novel differential RNA sequencing (dRNA-seq) approach on the RNA transcriptome of Salmonella Typhimurium to identify and locate transcriptional start sites on the chromosome. Based on this analysis, they reported that there were several-fold more promoters than had been previously identified and that numerous small regulatory RNA were expressed during early stationary phase growth with almost half being unique to the Salmonella genus. As RNA sequencing becomes more advanced, Westermann et al. (2012) have proposed the possibility of conducting dual RNA-seq where gene expression levels of both the pathogen and the host are assessed simultaneously.

This insight on concomitant pathogen-host gene expression responses is critical because it is now becoming apparent that Salmonella virulence and metabolism may be much more interlinked than previously thought (Rohmer et al., 2011). Consequently, emergence and prevalence of new Salmonella strains in poultry production may be as much a function of physiological fitness as it is of the classically identified colonization and pathogenic properties. Understanding the genetic factors and survival mechanisms under such conditions could provide better targets for development of more effective strategies to reduce Salmonella in poultry and poultry products. This would in turn lead to more focus on interventions that attack multiple and to some extent unrelated metabolic pathways. These approaches may prove to be a more effective means to not only restrict dissemination of Salmonella throughout poultry production but be more generally effective against multiple serotypes. This would have particular value for interventions such as Salmonella vaccines where their effectiveness is somewhat strain specific and there continues to be a need for multivalent-oriented vaccines (Wallis, 2001; Revolloco and Ferreira, 2012). It is anticipated that as more functional genetic/microarray and now RNA-seq studies are completed and combined with other comprehensive data generating technologies that a systems strategy similar to those proposed for other bacteria can be applied to the control of Salmonella for most of the steps involved in poultry production (De Keersmaecker et al., 2006; Park et al., 2013).

ACKNOWLEDGMENTS

This review was partially supported by a USDA-National Integrated Food Safety Initiative grant 2008-51110-04339 and a USDA-Sustainable Agriculture Research & Education (SARE) grant LS11-245 (Washington, DC) to author SCR, and by National Institutes of Health grant R21 AI063137 (Bethesda, MD) to author YMK.

REFERENCES


Downloaded from https://academic.oup.com/ps/article-abstract/92/9/2243/1560020 by guest on 24 December 2018