AEM
Journals.ASM.org

Gas Chromatography-Mass Spectrometry-Based Metabolite Profiling of *Salmonella enterica* Serovar Typhimurium Differentiates between Biofilm and Planktonic Phenotypes

Hui San Wong,[a,b] Garth L. Maker,[b,c] Robert D. Trengove,[c] Ryan M. O'Handley[a]

The University of Adelaide, School of Animal and Veterinary Science, Adelaide, South Australia, Australia[a]; Murdoch University, School of Veterinary and Life Sciences, Perth, Western Australia, Australia[b]; Murdoch University, Metabolomics Australia, Perth, Western Australia, Australia[c]

The aim of this study was to utilize gas chromatography coupled with mass spectrometry (GC-MS) to compare and identify patterns of biochemical change between *Salmonella* cells grown in planktonic and biofilm phases and *Salmonella* biofilms of different ages. Our results showed a clear separation between planktonic and biofilm modes of growth. The majority of metabolites contributing to variance between planktonic and biofilm supernatants were identified as amino acids, including alanine, glutamic acid, glycine, and ornithine. Metabolites contributing to variance in intracellular profiles were identified as succinic acid, putrescine, pyroglutamic acid, and *N*-acetylglutamic acid. Principal-component analysis revealed no significant differences between the various ages of intracellular profiles, which would otherwise allow differentiation of biofilm cells on the basis of age. A shifting pattern across the score plot was illustrated when analyzing extracellular metabolites sampled from different days of biofilm growth, and amino acids were again identified as the metabolites contributing most to variance. An understanding of biofilm-specific metabolic responses to perturbations, especially antibiotics, can lead to the identification of novel drug targets and potential therapies for combating biofilm-associated diseases. We concluded that under the conditions of this study, GC-MS can be successfully applied as a high-throughput technique for "bottom-up" metabolomic biofilm research.

Biofilm formation by *Salmonella* spp. on biotic and abiotic surfaces has profound consequences in many industries, as they act as continual sources of contamination and are notoriously difficult to eradicate (1–5). The regulatory network governing *Salmonella* biofilm formation is highly sophisticated; however, through the dedicated efforts of research groups worldwide, the picture is gradually becoming clearer. More recently, proteomic and transcriptomic studies have shown a global shift in metabolism when growth switches from sessile to a biofilm (6, 7). It is therefore highly plausible that phenotypic biofilm properties thought to be induced via biofilm-specific gene expression are in fact induced by differential expression of common metabolic pathways (7, 8).

The closest "omics" representation of phenotypes in a microorganism is metabolomics, or the study of the metabolome. Metabolomics incorporates both the analysis and identification of metabolites and allows researchers to monitor changes in metabolic profiles following perturbations to a system. Metabolomics is not new, but it has recently undergone a revival due to advances in analytical techniques, such as liquid chromatography-mass spectrometry (LC-MS) (9), nuclear magnetic resonance (NMR) (10), and gas chromatography coupled with mass spectrometry (GC-MS) (11, 12). The resurgence in metabolomics has subsequently provided an innovative high-throughput approach for research into the metabolic complexity of biofilms.

Current literature about biofilm metabolomics is limited and is mostly confined to studies using NMR (13–15). In this study, we utilized a GC-MS-based metabolomic approach to compare and identify patterns of biochemical change between planktonic and biofilm *Salmonella* cells. Biofilms were also grown for different lengths of time, and the same metabolomic approach was used to identify metabolic differences between *Salmonella* biofilms at different ages. We hypothesized that the metabolite profiles of planktonic and biofilm *Salmonella* are different from each other. We also hypothesized that the metabolite profiles of 3-, 5-, and 7-day-old biofilms are distinct between different days.

MATERIALS AND METHODS

***Salmonella* strain and culture conditions.** *Salmonella enterica* serovar Typhimurium ATCC 14028 was used as the reference strain in this study. *S.* Typhimurium was routinely cultured in Luria-Bertani (LB) medium and incubated aerobically at 37°C. Pure *Salmonella* colonies were verified by streaking the culture onto xylose-lysine-deoxycholate (XLD) agar (Becton Dickinson Difco) and incubation overnight at 37°C. Only red colonies with black centers were used (16).

Planktonic and biofilm culture conditions. A standardized microbial inoculum (25 ml of a culture concentration of 1.0×10^7 CFU/ml) was incubated in 50-ml tubes at 37°C for 24 h. This experiment was repeated to yield eight independent biological replicates for planktonic culture conditions. *Salmonella* biofilms were grown under conditions previously described by Wong and colleagues (5, 17). Biofilms were formed using the MBEC high-throughput assay (MBEC Innovotech, Canada), as previously described by Ceri and colleagues (18), with some modifications (5).

Received 17 November 2014 **Accepted** 27 January 2015

Accepted manuscript posted online 30 January 2015

Citation Wong HS, Maker GL, Trengove RD, O'Handley RM. 2015. Gas chromatography-mass spectrometry-based metabolite profiling of *Salmonella enterica* serovar Typhimurium differentiates between biofilm and planktonic phenotypes. Appl Environ Microbiol 81:2660–2666. doi:10.1128/AEM.03658-14.

Editor: G. Voordouw

Address correspondence to Hui San Wong, h.wong@mail.com.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.03658-14.

doi:10.1128/AEM.03658-14

The device consists of a 96-peg lid and a bottom ridged trough. A standardized microbial inoculum (25 ml of a culture concentration of 1.0×10^7 CFU/ml) was incubated in the device at 37°C on a rocking platform (Bio-Line platform rocker, model 4100; Edwards Instrument Company, New South Wales, Australia) for 24 h. This experiment was repeated to yield eight independent biological replicates for each condition ($n = 8$).

Chemicals. MilliQ water was obtained from a Millipore Milli-Q Synthesis A10 purification system. All other chemicals were purchased from Sigma-Aldrich (Switzerland) at the highest purity available unless otherwise stated.

Extracellular-metabolite preparation. Medium controls (10% LB with no sodium chloride) and planktonic and biofilm culture supernatants were filtered through a 0.2-μm membrane (Fast PES filter unit; Nalgene). A sample aliquot of 100 μl was mixed with 5 μg of ribitol (adonitol; minimum, 99%) to serve as an internal standard, made up to 300 μl with MilliQ H_2O and frozen at −80°C. Samples were freeze-dried overnight (Labconco FreeZone Plus 2.5-liter benchtop; Cascade Freeze Dry Systems) and stored at −80°C prior to analysis.

Intracellular-metabolite preparation. Planktonic cultures were centrifuged at 4,000 rpm for 30 min at 4°C (Allegra X-15R centrifuge; Beckman Coulter), the supernatant was removed, and the pellet was transferred into a clean tube and freeze-dried overnight. The following extraction was carried out on the lyophilized pellet: 500 μl of MilliQ H_2O containing 5 μg of ribitol was added to the pellet, vortexed to dissolve the pellet, sonicated for 5 min, vortexed for 30 s, sonicated for 5 min, frozen in liquid nitrogen, thawed on ice, vortexed for 30 s, and centrifuged (5415R centrifuge; Eppendorf, Germany; 13,200 rpm; 10 min; 4°C), and the supernatant was transferred into another clean tube. The extraction procedure was performed on the pellet twice thereafter using absolute methanol (methanol HPLC; RCI Labscan Limited, Thailand). All supernatants were pooled and placed in a rotary vacuum concentrator (Concentrator Plus AG 22331; Eppendorf, Germany) for 2 h at room temperature to remove the methanol, and the remaining supernatant was freeze-dried overnight.

The peg lids with biofilms were rinsed with 0.9% saline for 1 min to remove planktonic bacteria and then placed in a 96-well plate containing 200 μl of MilliQ H_2O per well and sonicated on high for 5 min (Bransonic 220; Branson Consolidated Ultrasonic Pty Ltd., Australia). The biofilm cells were centrifuged at 4,000 rpm for 30 min at 4°C, the supernatant was removed, and the pellet was transferred into a clean tube and freeze-dried overnight. Extractions were performed on the lyophilized biofilm pellet as described for planktonic cultures. All the supernatants were pooled and placed in a rotary vacuum concentrator for 2 h at room temperature to remove the methanol, and the remaining supernatant was freeze-dried overnight.

Sample derivatization. Lyophilized samples were resuspended in 20 μl of methoxyamine hydrochloride in pyridine (20 mg/ml) and mixed for 90 min at 30°C and 1,200 rpm (Thermomixer Comfort; Eppendorf, Germany). *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) (40 μl) was added to each sample and mixed for 30 min at 37°C, with shaking at 1,200 rpm for the first minute only. The derivatized samples were transferred into a 2-ml GC vial and tightly capped.

Gas chromatography-mass spectrometry analysis. The GC-MS analysis method used in this study has previously been used to generate metabolite profiles of *Stagonospora nodorum* (19). Briefly, the derivatized samples were analyzed using an Agilent 6890 gas chromatograph system coupled to an Agilent 5973 mass selective detector (Agilent Technologies Pty Ltd.). The fused silica capillary column was a FactorFour VF-5ms with dimensions of 30 m by 0.25 mm by 0.25 μm plus a 10-m EZ-Guard (Agilent Technologies). Ultra-high-purity helium (BOC Gases, Australia) was used as the carrier gas.

The derivatized samples were injected at 1 μl using a 20:1 split ratio for the supernatant and splitless injection for intracellular metabolites. The inlet temperature was set to 230°C. The initial oven temperature was set at 70°C, increasing 1°C per min for 5 min before increasing to a final temperature of 330°C at 5.63°C/min with a 10-min hold time. The helium flow rate was retention time locked to elute D-mannitol at 30.6 min. The mass spectrometer was set to a scan-monitoring range of m/z 40 to 600 at 1 scan/s.

Data processing and analysis. Mass spectra and chromatograms were normalized to a ribitol internal standard and processed using AnalyzerPro v2.2.07 (SpectralWorks Ltd., Runcorn, United Kingdom) and Unscrambler v9.8 (Camo Software, Norway). Principal-component analysis (PCA) was performed subsequent to $\log_{10}(x + 1)$ transformation of the normalized abundance data. Mass spectral similarity searches were performed using NIST MS search (NIST Mass Spectral Database, version 2.0f). All other data were analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Groups were compared using 1-way analysis of variance (ANOVA), followed by Dunnett's multiple-comparison test. Data were expressed as the mean normalized peak area ± standard error of the mean (SEM), and differences with a P value of <0.05 were considered significant.

RESULTS

Extracellular metabolites from planktonic and biofilm supernatants. PCA was used to model the data set (see the metabolite list [Data Set S1] in the supplemental material) and to determine the metabolites contributing most to the variance observed. The score plot generated by submitting extracellular metabolite profiles from medium control, biofilm, and planktonic samples to PCA is presented in Fig. 1A. Principal component 1 (PC-1) accounts for 63% of the variance in the data and clearly separates all three groups of samples. Metabolites affecting factor loading across PC-1 were identified, and the top eight are presented in Fig. 1B. Positive loadings were influenced most by alanine, glutamic acid, glycine, and ornithine, while negative loadings were influenced most by valine, glutaric acid, urea, and isoleucine. Amino acids are a constituent of Luria-Bertani broth (20), so to validate that the amino acids most affecting variance were not exclusively derived from the medium, the relative levels of the amino acids were compared between the different types of supernatant (Fig. 1C). Alanine, glycine, and glutamic acid were not detected in supernatants derived from biofilms but were present in both planktonic and medium samples. Valine and isoleucine were present in all three types of supernatants, but the peak areas of valine in both planktonic and biofilm samples were significantly different from the control, as was isoleucine in biofilm samples. Ornithine was detected only in planktonic samples and not biofilm or the controls.

Intracellular metabolite from planktonic and biofilm cells. The score plot generated by submitting metabolite profiles to PCA is presented in Fig. 2A and shows distinct separation between planktonic and biofilm cells. PC-1 accounts for 79% of the variance in the data, and PC-2 describes an additional 11% and also describes the spread within planktonic and biofilm samples. Metabolites affecting factor loading across PC-1 were identified, and the top eight are presented in Fig. 2B. Positive loadings were influenced most by succinic acid, putrescine, pyroglutamic acid, and *N*-acetylglutamic acid, while negative loadings were influenced most by glycerol, lactic acid, urea, and leucine.

Extracellular metabolites from biofilms grown for different numbers of days. The score plot generated by submitting biofilm supernatant metabolite profiles to PCA is presented in Fig. 3A. PC-1 accounts for 60% of the variance in the data, and PC-2 explains an additional 19% of the variance. A shifting trend across PC-1 was observed with different ages of biofilm supernatant. This trend was made prominent when samples were grouped together on the basis of the day (indicated by blue, red, and green) and was

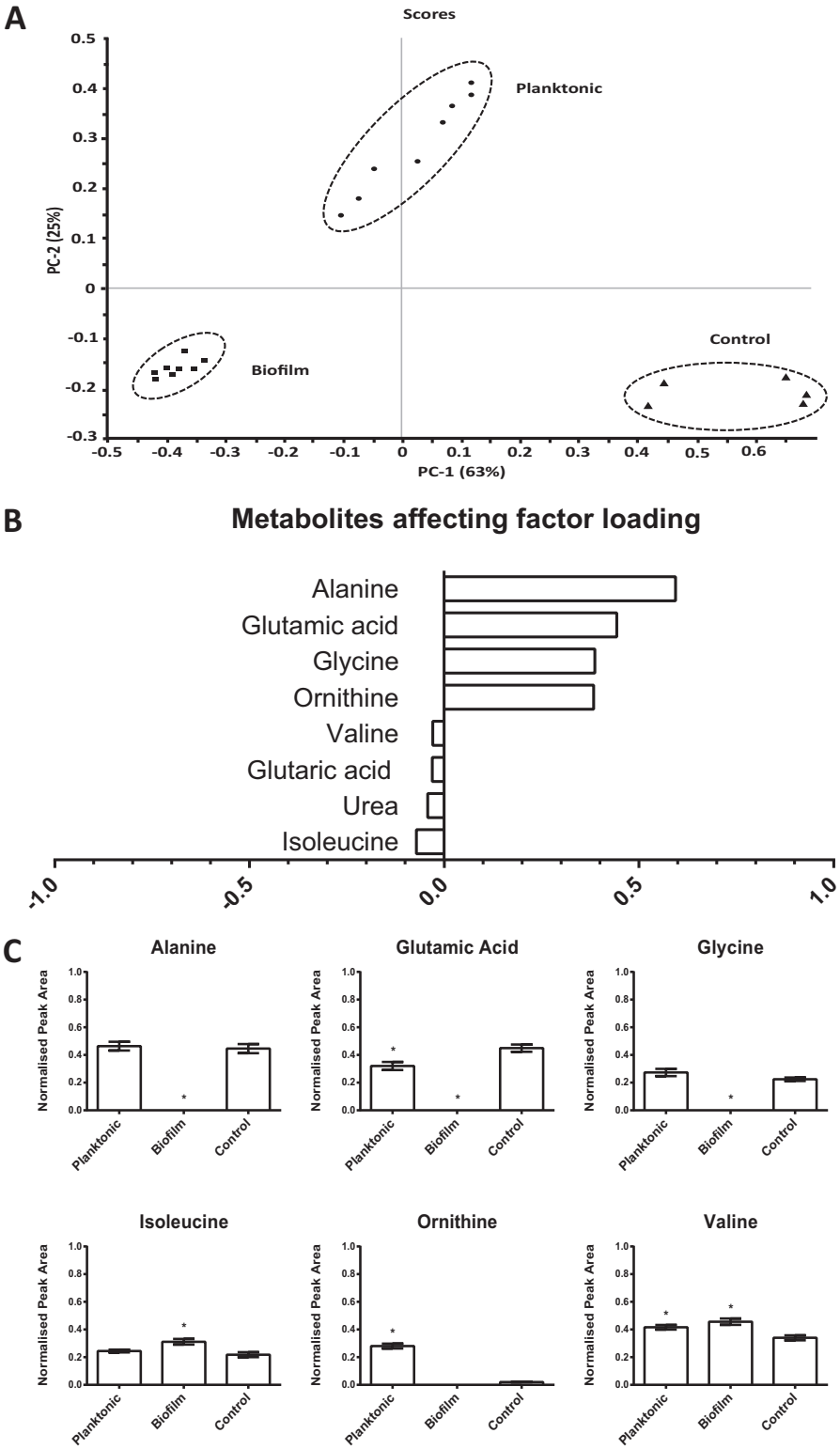

FIG 1 Extracellular metabolites from planktonic and biofilm supernatants and LB medium (control). (A) PCA score plot of metabolites from control, planktonic, and biofilm supernatants. (B) Metabolites most affecting factor loading across PC-1 when comparing profiles of planktonic and biofilm supernatants. (C) Peak areas, normalized to the internal standard, of the amino acids alanine, glycine, valine, isoleucine, glutamic acid, and ornithine in planktonic and biofilm supernatants and LB medium. Eight independent biological samples were analyzed for each condition, and five samples were analyzed for the control. The data are presented as the means ± SEM of normalized peak areas. All groups that were statistically different from the control ($P < 0.05$) are indicated by asterisks.

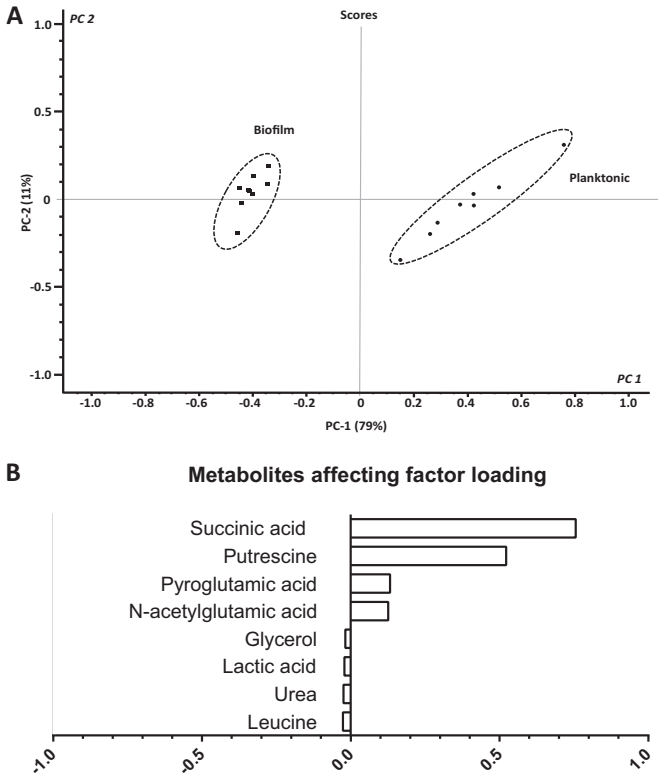

FIG 2 Intracellular metabolites from planktonic and biofilm cells. (A) PCA score plot of metabolites from planktonic and biofilm *Salmonella* Typhimurium cells. (B) Metabolites that had the greatest influence on the variance between samples when comparing profiles of planktonic and biofilm cells.

consistent for all samples except one outlying 7-day sample (as indicated). Metabolites affecting factor loading across PC-1 were identified, and the top eight are presented in Fig. 3B. Positive loadings were influenced most by valine, pyroglutamic acid, phenylalanine, and isoleucine, while negative loadings were influenced most by sorbitol, octadecanoic acid, (*Z*)-9-octadecenamide, and γ-hydroxyglutaric acid. The relative levels of the amino acids valine, pyroglutamic acid, phenylalanine, and isoleucine were compared between the various types of supernatant (Fig. 3C). The normalized peak areas of valine and isoleucine in 3-, 5- and 7-day-old biofilm supernatants were significantly different from the normalized peak areas of valine and isoleucine in the control medium ($P < 0.05$). The normalized peak areas of pyroglutamic acid and phenylalanine in 5- and 7-day-old biofilm supernatants were significantly different from the peak areas of pyroglutamic acid and phenylalanine in the control medium ($P < 0.05$). The normalized peak areas of 3-day-old supernatants were not significantly different from the controls for the last two amino acids. Therefore, while the amino acids of interest were present in the media, the significantly higher normalized peak area of each amino acid in the supernatant compared to controls indicates that they did not contribute greatly to the variance observed in our study.

Intracellular metabolites from biofilms grown for different numbers of days. The score plot generated by submitting 3-, 5-, and 7-day-old biofilm cell profiles to PCA is presented in Fig. 4. PC-1 accounts for 91% of the variance in the data, and PC-2 explains an additional 5% of the variance. PCA did not show any trends or clustering between the biofilm cells grown for different numbers of days, and as such, the biofilm cells could not be separated on the basis of age. As there was no evidence of separation on the score plot, metabolites affecting factor loading were not identified.

DISCUSSION

The present study was designed to compare and identify patterns of biochemical change between planktonic and biofilm growth in *Salmonella* Typhimurium and to determine if a GC-MS-based metabolomics approach can be successfully used as a high-throughput tool for biofilm research. To our knowledge, this is the first study that has evaluated metabolite profiles of planktonic and biofilm *Salmonella* Typhimurium cells using GC-MS.

Intensive research into the morphology, physiology, and genomics of biofilm formation has improved our knowledge about this remarkable phenomenon. Until recently, little attention has been directed toward the analysis of the metabolome in order to understand how sessile growth and biofilms differ at a metabolic level. Existing literature about biofilm metabolomics is limited to studies using NMR (13, 15), which is restricted by low-sensitivity measurements. MS is highly sensitive but less quantitative, and the inevitable use of chromatography to separate metabolites results in variations in the metabolome (21). As such, a combined application of NMR- and MS-based analytical techniques should yield a more comprehensive metabolite profile than applying each technique individually (22). Biofilm metabolomics will have to be carried out on a single, well-defined system due to the discrepancies in biofilms grown using different systems. *Salmonella* biofilm phenotypes depend on a variety of factors, including the isolate, nutrient source, and temperatures (2, 23, 24). While many research groups favor the use of flow cell systems for biofilm studies, our specific study used a static device known as the Calgary Biofilm Device, or commercially as the MBEC assay. The study of static biofilms is important, as not all natural biofilms exist as flow cell biofilms. For example, foodborne pathogens, such as *Salmonella*, more often develop biofilms in static environments, such as food-processing surfaces, equipment surfaces, and fresh produce (25, 26). In order to compare the metabolite profiles of biofilm and planktonic cells, it is crucial that all cells experience the same environment. Studies comparing flow cell biofilms to planktonic cultures have to take into consideration the accumulation of metabolic end products in planktonic growth, which does not occur in flow cell biofilms, as the medium is continually refreshed. The MBEC assay is an invaluable system for biofilm metabolomics research, as metabolites are not washed out but are instead able to accumulate. Appropriate selection of a biofilm system is also important when assessing biofilms of different ages, since biofilms often have a limited life span, disassembling as nutrients become exhausted and waste products accumulate (27).

It was expected that amino acids would contribute substantially to the observed variance, since amino acid metabolism is an important component of growth (28). The presence of urea, produced during amino acid decarboxylation (29), further supports our findings that amino acids play an important role in the life of a biofilm. The results from this study support existing research showing that amino acids are a crucial component in biofilm growth and development (6, 30, 31). Specifically, Valle et al. (30) demonstrated that valine production was a result of the metabolic

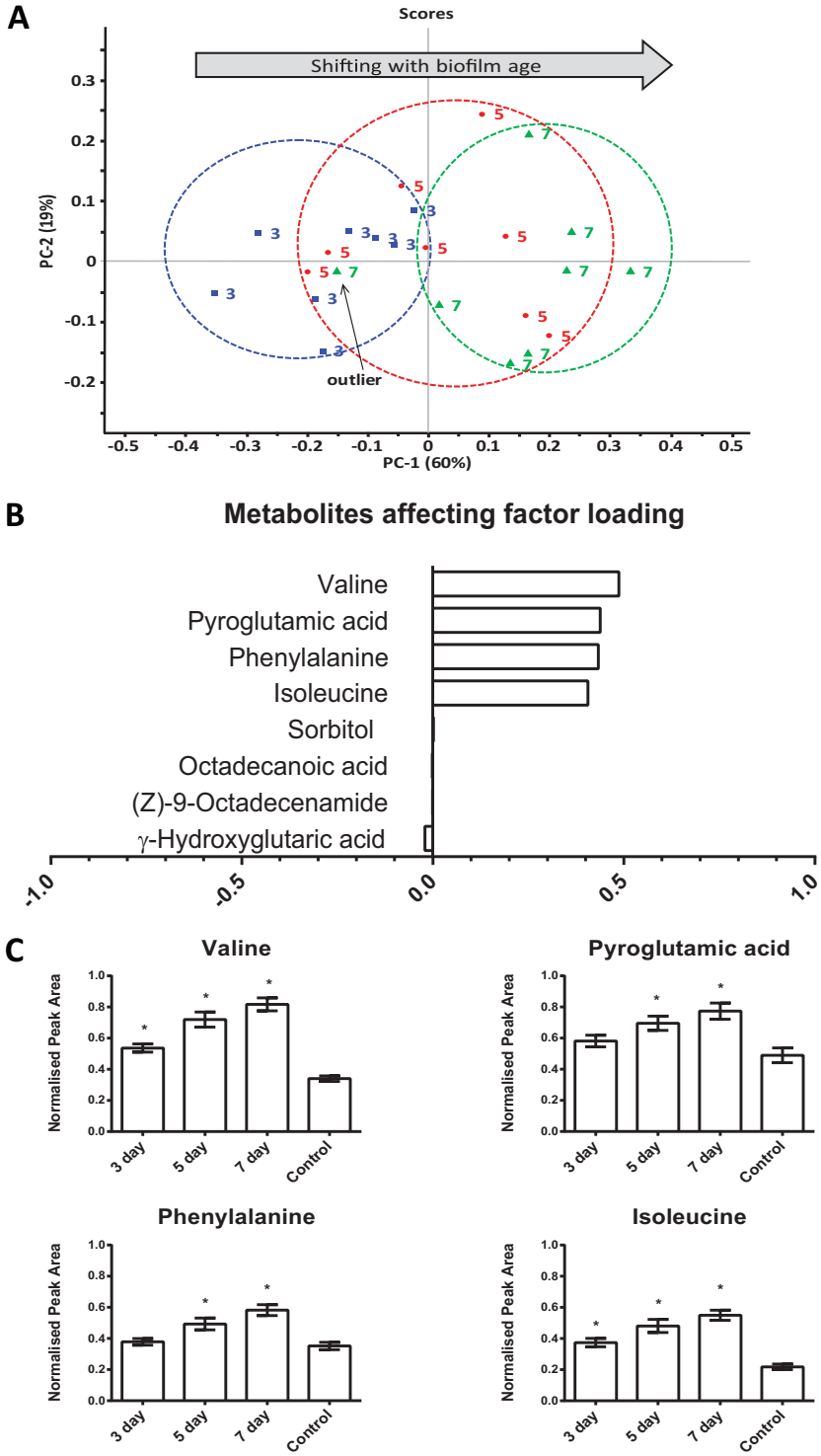

FIG 3 Extracellular metabolites from 3-, 5-, and 7-day-old biofilm supernatants and LB medium (control). (A) PCA score plot of metabolites from biofilm supernatants (3 [blue], 5 [red], and 7 [green] days old). (B) Metabolites most affecting factor loading across PC-1 when comparing profiles of biofilms at 3, 5, and 7 days. (C) Normalized peak areas of the amino acids valine, pyroglutamic acid, phenylalanine, and isoleucine in 3-, 5-, and 7-day-old supernatants and LB medium (control). Eight independent biological samples were analyzed for each day, and five samples were analyzed for the control. The data are presented as the means ± SEM of normalized peak areas. All groups that were statistically different from the control ($P < 0.05$) are indicated by asterisks.

changes occurring within high-density biofilm communities. Our findings showed an increasing level of amino acids in biofilm supernatant as the biofilm ages. Therefore, while amino acids may constitute an energy source in biofilms, they may also have another role as a signal for biofilm maturation and eventual disassembly. These findings corroborate studies that have determined that D-amino acids produced by *Bacillus subtilis* prevent biofilm formation and disrupt existing biofilms by causing the release of

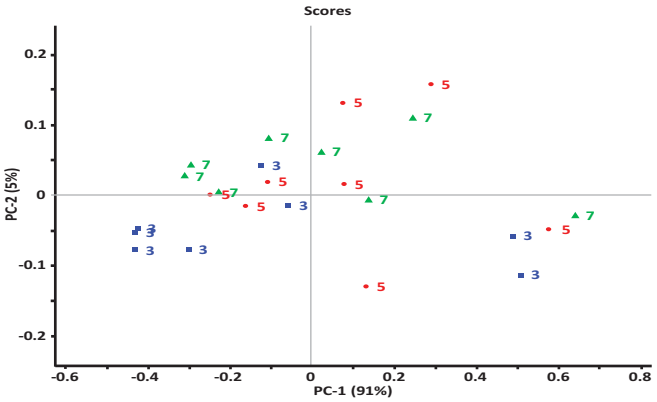

FIG 4 Intracellular metabolites from 3-, 5-, and 7-day-old biofilm cells (blue, red, and green, respectively). Shown is a PCA score plot of metabolites from the biofilm cells.

fibers connecting the cells within a biofilm (32). It has since been reported by the same group that self-produced norspermidine acts with D-amino acids to trigger biofilm disassembly (33). Although the D-amino acids identified by Kolodkin-Gal and colleagues were not an exact match to our findings, it would still be of considerable interest to evaluate other amino acids and their roles, both individually and synergistically, in biofilm development and dispersion.

Biofilms formed a tighter collection on the score plot than planktonic cells in both intra- and extracellular profiles. This was unexpected, as it is assumed that planktonic cells are homogeneous and therefore should have analogous metabolisms. The reason why biofilm cells formed a tighter grouping in this study is unknown; however, it does suggest that the metabolism of biofilms is more closely regulated than that of planktonic cells. The wide range of metabolites affecting factor loading in biofilm and planktonic cells was not surprising given that *Salmonella* metabolism is intricate, with more than 250 genes essential for *in vitro* growth in LB medium (34) and over 2,000 proteins with putative metabolic functions (35). While the precise roles of these metabolites in *Salmonella* metabolism and, more specifically, their relationship with biofilm development are of interest and an area for further research, they are beyond the scope of the current study.

While biofilm supernatants could be separated on the basis of age, it was interesting that intracellular samples were not separated on the same basis. It is well established that microenvironments exist within a biofilm (36), and thus, the metabolic activity of biofilm-associated cells under oxygen and nutrient stress will be very different from the metabolic activity of cells in oxygen- and nutrient-rich microenvironments. In addition to this, it is known that different types of cells exist within a biofilm. For example, persister cells are a dormant subpopulation of biofilm-associated cells that neither grow nor perish in the presence of antimicrobial agents due to limited metabolic activity (37–39). The metabolic diversity of cells within a biofilm of any age would therefore make it difficult to separate the samples on the basis of biofilm "age" alone. The sample preparation methods and the limits of detection of the analytical instrument utilized in this study can also be modified and compared in future studies to determine the most suitable technique for assessing intracellular biofilm metabolites.

In summary, we have demonstrated that a GC-MS-based metabolomics approach can be used to differentiate *Salmonella* biofilm and sessile growth in conjunction with identification of metabolites that cause variance. Our results have reinforced the importance of amino acids in biofilm growth and the significance of other metabolites that are utilized during biofilm development. Biochemical-pathway studies and different biofilm growth environments, combined with developments in extraction methods, instrumentation, platforms, databases, libraries, and statistical software, will propel this field of biofilm "omics" forward and increase our understanding of the biofilm metabolome.

ACKNOWLEDGMENTS

This work was supported by the Environmental Biotechnology CRC, Murdoch University, Perth, Western Australia, Australia, and Metabolomics Australia, Murdoch University, Perth, Western Australia, Australia.

We declare no conflict of interest.

REFERENCES

1. **Leriche V, Carpentier B.** 1995. Viable but nonculturable Salmonella typhimurium in single- and binary-species biofilms in response to chlorine treatment. J Food Prot **58:**1186–1191.
2. **Scher K, Romling U, Yaron S.** 2005. Effect of heat, acidification, and chlorination on Salmonella enterica serovar Typhimurium cells in a biofilm formed at the air-liquid interface. Appl Environ Microbiol **71:**1163–1168. http://dx.doi.org/10.1128/AEM.71.3.1163-1168.2005.
3. **Tabak M, Scher K, Hartog E, Romling U, Matthews KR, Chikindas ML, Yaron S.** 2007. Effect of triclosan on Salmonella typhimurium at different growth stages and in biofilms. FEMS Microbiol Lett **267:**200–206. http://dx.doi.org/10.1111/j.1574-6968.2006.00547.x.
4. **Mangalappalli-Illathu AK, Vidovic S, Korber DR.** 2008. Differential adaptive response and survival of Salmonella enterica serovar Enteritidis planktonic and biofilm cells exposed to benzalkonium chloride. Antimicrob Agents Chemother **52:**3669–3680. http://dx.doi.org/10.1128/AAC.00073-08.
5. **Wong HS, Townsend KM, Fenwick SG, Trengove RD, O'Handley RM.** 2010. Comparative susceptibility of planktonic and 3-day-old Salmonella Typhimurium biofilms to disinfectants. J Appl Microbiol **108:**2222–2228. http://dx.doi.org/10.1111/j.1365-2672.2009.04630.x.
6. **Hamilton S, Bongaerts RJ, Mulholland F, Cochrane B, Porter J, Lucchini S, Lappin-Scott HM, Hinton JC.** 2009. The transcriptional programme of Salmonella enterica serovar Typhimurium reveals a key role for tryptophan metabolism in biofilms. BMC Genomics **10:**599. http://dx.doi.org/10.1186/1471-2164-10-599.
7. **White AP, Weljie AM, Apel D, Zhang P, Shaykhutdinov R, Vogel HJ, Surette MG.** 2010. A global metabolic shift is linked to Salmonella multicellular development. PLoS One **5:**e11814. http://dx.doi.org/10.1371/journal.pone.0011814.
8. **Steenackers H, Hermans K, Vanderleyden J, De Keersmaecker SCJ.** 2012. Salmonella biofilms: an overview on occurrence, structure, regulation and eradication. Food Res Int **45:**502–531. http://dx.doi.org/10.1016/j.foodres.2011.01.038.
9. **Buchholz A, Hurlebaus J, Wandrey C, Takors R.** 2002. Metabolomics: quantification of intracellular metabolite dynamics. Biomol Eng **19:**5–15. http://dx.doi.org/10.1016/S1389-0344(02)00003-5.
10. **Griffin JL.** 2003. Metabonomics: NMR spectroscopy and pattern recognition analysis of body fluids and tissues for characterisation of xenobiotic toxicity and disease diagnosis. Curr Opin Chem Biol **7:**648–654. http://dx.doi.org/10.1016/j.cbpa.2003.08.008.
11. **Roessner U, Wagner C, Kopka J, Trethewey RN, Willmitzer L.** 2000. Technical advance: simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. Plant J **23:**131–142. http://dx.doi.org/10.1046/j.1365-313x.2000.00774.x.
12. **Fiehn O, Kopka J, Dormann P, Altmann T, Trethewey RN, Willmitzer L.** 2000. Metabolite profiling for plant functional genomics. Nat Biotechnol **18:**1157–1161. http://dx.doi.org/10.1038/81137.
13. **Yeom J, Shin JH, Yang JY, Kim J, Hwang GS.** 2013. (1)H NMR-based metabolite profiling of planktonic and biofilm cells in Acinetobacter baumannii 1656-2. PLoS One **8:**e57730. http://dx.doi.org/10.1371/journal.pone.0057730.
14. **Wu XH, Yu HL, Ba ZY, Chen JY, Sun HG, Han BZ.** 2010. Sampling

methods for NMR-based metabolomics of Staphylococcus aureus. Biotechnol J **5:**75–84. http://dx.doi.org/10.1002/biot.200900038.
15. **Gjersing EL, Herberg JL, Horn J, Schaldach CM, Maxwell RS.** 2007. NMR metabolomics of planktonic and biofilm modes of growth in Pseudomonas aeruginosa. Anal Chem **79:**8037–8045. http://dx.doi.org/10.1021/ac070800t.
16. **Taylor WI.** 1965. Isolation of shigellae. I. Xylose lysine agars; new media for isolation of enteric pathogens. Am J Clin Pathol **44:**471–475.
17. **Wong HS, Townsend KM, Fenwick SG, Maker G, Trengove RD, O'Handley RM.** 2010. Comparative susceptibility of *Salmonella typhimurium* biofilms of different ages to disinfectants. Biofouling **26:**859–864. http://dx.doi.org/10.1080/08927014.2010.527959.
18. **Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A.** 1999. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J Clin Microbiol **37:**1771–1776.
19. **Tan KC, Trengove RD, Maker GL, Oliver RP, Solomon PS.** 2009. Metabolite profiling identifies the mycotoxin alternariol in the pathogen Stagonospora nodorum. Metabolomics **5:**330–335. http://dx.doi.org/10.1007/s11306-009-0158-2.
20. **Sezonov G, Joseleau-Petit D, D'Ari R.** 2007. Escherichia coli physiology in Luria-Bertani broth. J Bacteriol **189:**8746–8749. http://dx.doi.org/10.1128/JB.01368-07.
21. **Zhang B, Powers R.** 2012. Analysis of bacterial biofilms using NMR-based metabolomics. Future Med Chem **4:**1273–1306. http://dx.doi.org/10.4155/fmc.12.59.
22. **Booth SC, Workentine ML, Wen J, Shaykhutdinov R, Vogel HJ, Ceri H, Turner RJ, Weljie AM.** 2011. Differences in metabolism between the biofilm and planktonic response to metal stress. J Proteome Res **10:**3190–3199. http://dx.doi.org/10.1021/pr2002353.
23. **Zogaj X, Nimtz M, Rohde M, Bokranz W, Romling U.** 2001. The multicellular morphotypes of Salmonella typhimurium and Escherichia coli produce cellulose as the second component of the extracellular matrix. Mol Microbiol **39:**1452–1463. http://dx.doi.org/10.1046/j.1365-2958.2001.02337.x.
24. **Giaouris E, Chorianopoulos N, Nychas GJ.** 2005. Effect of temperature, pH, and water activity on biofilm formation by Salmonella enterica enteritidis PT4 on stainless steel surfaces as indicated by the bead vortexing method and conductance measurements. J Food Prot **68:**2149–2154.
25. **Annous BA, Solomon EB, Cooke PH, Burke A.** 2005. Biofilm formation by Salmonella spp. on cantaloupe melons. J Food Saf **25:**276–287. http://dx.doi.org/10.1111/j.1745-4565.2005.00024.x.
26. **Patel J, Sharma M.** 2010. Differences in attachment of Salmonella enterica serovars to cabbage and lettuce leaves. Int J Food Microbiol **139:**41–47. http://dx.doi.org/10.1016/j.ijfoodmicro.2010.02.005.
27. **Karatan E, Watnick P.** 2009. Signals, regulatory networks, and materials that build and break bacterial biofilms. Microbiol Mol Biol Rev **73:**310–347. http://dx.doi.org/10.1128/MMBR.00041-08.
28. **Bayne HG, Stokes JL.** 1961. Amino acid metabolism of salmonellae. J Bacteriol **81:**126–129.
29. **Geornaras I, Dykes GA, von Holy A.** 1995. Biogenic amine formation by poultry-associated spoilage and pathogenic bacteria. Lett Appl Microbiol **21:**164–166. http://dx.doi.org/10.1111/j.1472-765X.1995.tb01032.x.
30. **Valle J, Da Re S, Schmid S, Skurnik D, D'Ari R, Ghigo JM.** 2008. The amino acid valine is secreted in continuous-flow bacterial biofilms. J Bacteriol **190:**264–274. http://dx.doi.org/10.1128/JB.01405-07.
31. **Workentine ML, Harrison JJ, Weljie AM, Tran VA, Stenroos PU, Tremaroli V, Vogel HJ, Ceri H, Turner RJ.** 2010. Phenotypic and metabolic profiling of colony morphology variants evolved from Pseudomonas fluorescens biofilms. Environ Microbiol **12:**1565–1577. http://dx.doi.org/10.1111/j.1462-2920.2010.02185.x.
32. **Kolodkin-Gal I, Romero D, Cao S, Clardy J, Kolter R, Losick R.** 2010. D-amino acids trigger biofilm disassembly. Science **328:**627–629. http://dx.doi.org/10.1126/science.1188628.
33. **Kolodkin-Gal I, Cao S, Chai L, Bottcher T, Kolter R, Clardy J, Losick R.** 2012. A self-produced trigger for biofilm disassembly that targets exopolysaccharide. Cell **149:**684–692. http://dx.doi.org/10.1016/j.cell.2012.02.055.
34. **Knuth K, Niesalla H, Hueck CJ, Fuchs TM.** 2004. Large-scale identification of essential Salmonella genes by trapping lethal insertions. Mol Microbiol **51:**1729–1744. http://dx.doi.org/10.1046/j.1365-2958.2003.03944.x.
35. **McClelland M, Sanderson KE, Spieth J, Clifton SW, Latreille P, Courtney L, Porwollik S, Ali J, Dante M, Du F, Hou S, Layman D, Leonard S, Nguyen C, Scott K, Holmes A, Grewal N, Mulvaney E, Ryan E, Sun H, Florea L, Miller W, Stoneking T, Nhan M, Waterston R, Wilson RK.** 2001. Complete genome sequence of Salmonella enterica serovar Typhimurium LT2. Nature **413:**852–856. http://dx.doi.org/10.1038/35101614.
36. **Hamilton WA.** 1987. Biofilms: microbial interactions and metabolic activities. Cambridge University Press, Cambridge, United Kingdom.
37. **Lewis K.** 2005. Persister cells and the riddle of biofilm survival. Biochemistry **70:**267–274.
38. **Keren I, Shah D, Spoering A, Kaldalu N, Lewis K.** 2004. Specialized persister cells and the mechanism of multidrug tolerance in Escherichia coli. J Bacteriol **186:**8172–8180. http://dx.doi.org/10.1128/JB.186.24.8172-8180.2004.
39. **Lewis K.** 2007. Persister cells, dormancy and infectious disease. Nat Rev Microbiol **5:**48–56. http://dx.doi.org/10.1038/nrmicro1557.