The secret lives of single cells

Thomas K. Wood

Department of Chemical Engineering, Pennsylvania State University, University Park, Pennsylvania 16802-4400, USA.

Summary

Looking back fondly on the first 15 years of *Microbial Biotechnology*, a trend is emerging that biotechnology is moving from studies that focus on whole-cell populations, where heterogeneity exists even during robust growth, to those with an emphasis on single cells. This instils optimism that insights will be made into myriad aspects of bacterial growth in communities.

*Microbial Biotechnology* is special to me as my group published the first two research papers in the new journal: ‘*Pseudomonas aeruginosa* PAO1 virulence factors and poplar tree response in the rhizosphere’, vol. 1 pages: 17–29, 24 August 2007 (cited 73 times to date) (Attila et al., 2008) and ‘Metabolic engineering to enhance bacterial hydrogen production’, vol. 1 pages: 30-39, 24 August 2007 (cited 153 times to date) (Maeda et al., 2008). In the first issue of the second year, we published another manuscript, ‘Indole and 7-hydroxyindole diminish *Pseudomonas aeruginosa* virulence’, vol 2 pages: 75–90, 22 December 2008 (cited 188 times to date) (Lee et al., 2009). I am proud of these three manuscripts and congratulate the editors on the successful launch of their journal. Their vision was to create a journal where high-quality work could receive rapid review and dissemination, and they achieved their aims.

In two of these pioneering manuscripts, we used DNA microarrays to determine the transcriptome of the whole population of *P. aeruginosa* cells responding either to poplar tree roots (Attila et al., 2008) or to indole (Lee et al., 2009). By measuring the whole-population transcriptome, we discovered seven novel *P. aeruginosa* virulence genes this organism uses with plants and discovered indole is an inter-species signal from *Escherichia coli* that quenches quorum signalling of non-indole-synthesizing *P. aeruginosa* cells without affecting their growth. This led to numerous discoveries such as indole (i) prevents the resuscitation of *P. aeruginosa* persister cells (Zhang et al., 2019), (ii) kills bacterial and archaeal persister cells (Hu et al., 2015; Kwan et al., 2015a; Lee et al., 2016; Megaw and Gilmore, 2017; Li et al., 2019; Song et al., 2019; Manoharan et al., 2020; Sun et al., 2020; Yam et al., 2020), (iii) helps prevent infections as an *interkingdom* signal in the gastrointestinal tract by tightening epithelial cell junctions (Bansal et al., 2010; Shimada et al., 2013), (iv) regulates ageing in mice (Powell et al., 2020) and (v) influences brain development via the aryl-hydrocarbon receptor (Spichak et al., 2021). Therefore, these early publications that made use of whole-population studies in *Microbial Biotechnology* had a sizeable impact.

It is fascinating now that the field is moving rapidly from studying whole-cell populations, as we did in the early *Microbial Biotechnology* manuscripts, to determining the transcriptome of single bacterial cells. The logical progression was from DNA microarrays of whole-cell populations to RNA-seq of whole-cell populations to RNA-seq of single cells. For the single-cell studies, to date, there have been four main contributions of this RNA-seq technique in bacteria and one single-molecule fluorescence in situ hybridization (FISH) contribution. The first published method (25 May 2020, PETRI-seq) was that of Blatmann et al. (2020) which identified 200 *E. coli* transcripts per exponentially growing cell as well as identified rare prophage induction in *Staphylococcus aureus* cells. The second published method (17 August 2020, MATQ-seq) was that of Imdahl et al. (2020) which quantified the impact of growth on expression of 170 *Salmonella enterica* serovar Typhimurium genes and 102 *Pseudomonas aeruginosa* genes. Next, Kuchina et al. (2020) (17 December 2020, microSPLIT) were able to detect 235 transcripts/cell for *E. coli* and 397 transcripts/cell for *B. subtilis* at different growth stages. Most recently (10 March 2021), McNulty et al. (McNulty et al., 2021) sequenced 15,000 cells and detected 265 transcripts/B. subtilis cells and 149 transcripts/E. coli cell. In a different approach, Dar et al. (2021) used par-seqFISH to spatially resolve and quantify hundreds of transcripts...
within a single \textit{P. aeruginosa} biofilm cell (25 February 2021). Clearly, this area is moving rapidly, but it already allows one to determine which genes are expressed in a single cell as well as to identify in which specific part of the cell that the expression occurs.

The impact of discerning the response of single cells will be huge. For example, already for persister cells, single-cell observations have led to the discovery that the mechanism by which persister cells form as well as how they resuscitate is based on their active ribosome content. Persister cells are a subpopulation of cells which arise due to stress (e.g. antibiotic, nutritive, oxidative) and weather the stress by becoming dormant (Wood and Song, 2020). Specifically, single-cell studies were used to discover that persister cells become dormant by mothballing their protein synthesis machinery by making 100S ribosome dimers based on (p)ppGpp and cAMP signalling and through the actions of the ribosome-inactivating proteins ribosome-associated inhibitor A (RaiA), ribosome modulation factor (RMF) and ribosome hibernation-promoting factor (Hpf) (Kim et al., 2018; Song and Wood, 2020; Wood and Song, 2020). Moreover, dormant persister cells resuscitate, upon the removal of the stress and the presence of nutrients, by activating the mothballed ribosomes via nutrient sensing through membrane chemotaxis proteins and sugar transport proteins, which leads to a reduction in the (p)ppGpp and cAMP signals and activation of HIIX (Yamasaki et al., 2020). When they wake, the formerly dormant cells grow exponentially like wild-type cells (Kim et al., 2018). This ribosome-based mechanism was completely obscured by the previous whole-cell population studies that tried to evaluate behaviour based on population lag times. Critically, since persister cells reconstitute infections, their mechanism of formation and resuscitation is important since over two million people a year die from bacterial infections currently, and this total is projected to increase to 10 million by 2050 at a cost of $100 trillion (Thappeta et al., 2020). Furthermore, although inhibiting cAMP and (p)ppGpp would likely have pleiotropic effects, these mechanistic insights suggest RaiA, RMF (conserved in gammaproteobacterial), Hpf (conserved in bacteria and all domains of life), and HIIX (conserved GTpase from bacteria to humans) are excellent targets to prevent persistence; i.e., if cells fail to mothball their ribosomes, they remain susceptible to antimicrobials.

In a similar manner, the single-cell approach should lead to breakthroughs regarding our understanding how toxin/antitoxin (TA) systems function (Bruggeman, 2021, unpublished data) and how antibiotic resistance arises. TA systems are categorized into seven main types based on the function of the antitoxin (Wang et al., 2020), and multiple TA systems are found in almost all genomes (Yamaguchi et al., 2011). Although prevalent, their role in cell physiology is somewhat controversial, although TA systems have a clear role in phage inhibition (Pecota and Wood, 1996; Hazan and Engelberg-Kulka, 2004; Fineran et al., 2009), plasmid stabilization (Ogura and Hiraga, 1983), mobile genetic element stabilization (Wozniak and Waldor, 2009; Soutourina, 2019), plasmid copy number control (Ni et al., 2021), and biofilm formation (Ren et al., 2004; Kim et al., 2009).

As a controversial and well-studied example, the type II TA system MqsR/MqsA was first identified as active in \textit{E. coli} biofilms (Ren et al., 2004) and shown, based on both deletion and overexpression studies, to protect \textit{E. coli} from the bile acid it encounters in the gastrointestinal tract (Kwan et al., 2015b) as well as to take part in the general stress response by regulating the master regulator of the stress response, sigma factor RpoS (Wang et al., 2011). MqsR/MqsA have also been found to have an effect in non-\textit{E. coli} systems including copper stress (Merfa et al., 2016), vesicles (Santiago et al., 2016), and biofilm formation (Lee et al., 2014) in \textit{Xylella fastidiosa} as well as biofilm formation in \textit{Pseudomonas fluorescens} (Wang et al., 2019), and persistence and biofilm formation in \textit{Pseudomonas putida} (Sun et al., 2017).

However, two recent reports questioned the impact of MqsR/MqsA on cell physiology. The Van Melderen group claimed there was no induction of \textit{mqsRA} and no phenotype upon deleting \textit{mqsRA} during stress (Fraikin et al., 2019). A few months later, the Laub group invalidated the claim of no transcription response during stress by showing \textit{mqsRA} was induced dramatically (181 fold) during amino acid stress and during oxidative stress (90 fold) (LeRoux et al., 2020). The Laub group also failed to find a phenotype for MqsR/MqsA during stress (LeRoux et al., 2020), although bile acid stress was not investigated, biofilms were not investigated, and their results are flawed in that they relied on the use of a TA system deletion strain that has many non-related mutations (large chromosomal inversions) (Goormaghtigh et al., 2018). Note the use of TA system deletion strains with coding errors have led to notorious errors in the field that have led to three retractions, based on the errors we have described (Wood and Song, 2020). Critically, both of these studies that failed to find a phenotype with MqsR/MqsA used whole-population studies and therefore probably missed MqsR toxin expression in a subpopulation of cells. MqsR is very toxic; i.e., deletion of the antitoxin gene is lethal (Baba et al., 2006), so it is likely only a few molecules of this powerful RNase enzyme are produced and are produced in a subpopulation of cells (Bruggeman, 2021, unpublished data).

Similarly, breakthroughs in understanding how antibiotic resistance arises will likely be achieved by studying single cells. Currently, it is clear antibiotic resistance
arises from a series of mutations in non-dormant bacteria that gradually change lag phase (Santi et al., 2021) and metabolism (Lopatkin et al., 2021). However, so far, these studies are limited to studying whole-cell populations rather than single cells. Just as whole-population studies were unable to discern the mechanism of persister formation and resuscitation (e.g. by focussing on growth lags of large populations of cells), single-cell studies should enable the mechanism of antibiotic resistance to be determined more robustly by following changes in a single cell.

Therefore, one can be sanguine about the mechanisms that single-cell studies will provide in the next 10 years. Compared to coarse microarray studies on whole populations of cells, such as one for E. coli biofilm cells developed on glass wool (Ren et al., 2004), which led to the discovery of the TA systems Hha/TomB (Marimon et al., 2016) and MqsR/MqsA (Brown et al., 2009; Wang et al., 2011; Wang et al., 2013), single-cell sequencing and other single-cell techniques (e.g. proteomics, metabolomics, phenotype mapping, microscopy) are expected to provide myriad insights into the secret lives of single cells, including how biofilms form and function, how persister cells arise in stressed clonal populations, how TA systems impact cell physiology, how antibiotic resistance occurs, and how various protection systems are invoked and interact for lytic and temperate phages.

Acknowledgements

This work was supported by funds derived from the Biotechnology Endowed Professorship for TKW at Pennsylvania State University. The idea that toxin MqsR and other toxins are produced only in a subpopulation of cells is that of Professor Frank J. Bruggeman (Vrije Universiteit Amsterdam).

Funding information

No funding information provided.

Conflict of interest

None declared.

References

Attila, C., Ueda, A., Cirillo, S.L.G., Cirillo, J.D., Chen, W., and Wood, T.K. (2008) Pseudomonas aeruginosa PA01 virulence factors and poplar tree resistance in the rhizosphere. Microb Biotechnol 1: 17–29.
Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., et al. (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Molec Sys Biol 2: 2006.0008.
Bansal, T., Alaniz, R.C., Wood, T.K., and Jayaraman, A. (2010) The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation. Proc Natl Acad Sci USA 107: 228–233.
Blattman, S.B., Jiang, W., Oikonomou, P., and Tavazoie, S. (2020) Prokaryotic single-cell RNA sequencing by in situ combinatorial indexing. Nat Microbiol 5: 1192–1201.
Brown, B.L., Grigorii, S., Kim, Y., Arruda, J.M., Davenport, A., Wood, T.K., et al. (2009) Three dimensional structure of the MqsR:MqsA complex: a novel toxin:antitoxin pair comprised of a toxin homologous to RelE and an antitoxin with unique properties. PLoS Pathog 5: e1000706.
Dar, D., Dar, N., Cai, L., and Newman, D. K. (2021) In situ single-cell activities of microbial populations revealed by spatial transcriptomics. bioRxiv: 2021.2002.2024.432792.
Fineran, P.C., Blower, T.R., Foulds, I.J., Humphreys, D.P., Lilley, K.S., and Salmond, G.P.C. (2009) The phage abortive infection system, ToxIN, functions as a protein–RNA toxin–antitoxin pair. Proc Natl Acad Sci USA 106: 894–899.
Fraikin, N., Rousseau, C.J., Goeders, N., and Van Melderen, L. (2019) Reassessing the role of the type II MqsRA toxin-antitoxin system in stress response and biofilm formation: mqsA is transcriptionally uncoupled from mqsR. mBio 10: e02678–e2619.
Goormaghtigh, F., Fraikin, N., Putrinš, M., Hallaert, T., Hauryliuk, V., Garcia-Pino, A., et al. (2018) Reassessing the role of type II toxin-antitoxin systems in formation of Escherichia coli type II persister cells. mBio 9: e00640–e618.
Hazan, R., and Engelberg-Kulka, H. (2004) Escherichia coli mazEF-mediated cell death as a defense mechanism that inhibits the spread of phage P1. Mol Genet Genomics 272: 227–234.
Hu, Y., Kwan, B.W., Osbourne, D.O., Benedik, M.J., and Wood, T.K. (2015) Toxin YafQ increases persister cell formation by reducing indole signalling. Environ Microbiol 17: 1275–1285.
Imdahl, F., Vafadarenejad, E., Homberger, C., Saliba, A.-E., and Vogel, J. (2020) Single-cell RNA-sequencing reports growth-condition-specific global transcriptomes of individual bacteria. Nat Microbiol 5: 1202–1206.
Kim, J.-S., Yamasaki, R., Song, S., Zhang, W., and Wood, T.K. (2018) Single cell observations show persister cells wake based on ribosome content. Environ Microbiol 20: 2085–2098.
Kim, Y., Wang, X., Ma, Q., Zhang, X.-S., and Wood, T.K. (2009) Toxin-antitoxin systems in Escherichia coli influence biofilm formation through YfgK (TabA) and fimbiae. J Bacteriol 191: 1258–1267.
Kuchina, A., Brettner, L.M., Paleologu, L., Roco, C.M., Rosenberg, A.B., Carignano, A., et al. (2020) Microbial single-cell RNA sequencing by split-pool barcoding. Science 371: eaba5257.
Kwan, B.W., Lord, D.M., Peti, W., Page, R., Benedik, M.J., and Wood, T.K. (2015b) The MqsR:MqsA toxin/antitoxin system protects Escherichia coli during bile acid stress. Environ Microbiol 17: 3169–3181.
Kwan, B.W., Osbourne, D.O., Hu, Y., Benedik, M.J., and Wood, T.K. (2015a) Phosphodiesterase DosP increases...
persistence by reducing cAMP which reduces the signal
indole. Biotechnol Bioengr 112: 588–600.
Lee, J., Attiia, C., Cirillo, S.L.G., Cirillo, J.D., and Wood, T.K.
(2009) Indole and 7-hydroxyindole diminish Pseudomonas
aeruginosa virulence. Microbiol Biotech 2: 75–90.
Lee, J.-H., Kim, Y.-G., Gwon, G., Wood, T.K., and Lee, J.
(2016) Halogenated indoles eradicate bacterial persister
cells and biofilms. AMB Express 6: 123.
Lee, M.W., Tan, C.C., Rogers, E.E., and Stenger, D.C.
(2014) Toxin-antitoxin systems mqsR/ygiT and dinJ/relE
of Xylella fastidiosa. Physiol Molec Plant Path 87: 59–68.
LeRoux, M., Culviner, P.H., Liu, Y.J., Littlehale, M.L., and Laub,
M.T. (2020) Stress induces the transcription of toxin-antitoxin
systems but does not activate toxin. Mol Cell 79: 1–13.
Li, Y., Liu, B., Guo, J., Cong, H., He, S., Zhou, H., et al.
(2019) L-Tryptophan represses persister formation via
inhibiting bacterial motility and promoting antibiotics
absorption. Fut Microbiol 14: 757–771.
Lopatin, A.J., Bening, S.C., Manson, A.L., Stokes, J.M.,
Kohanski, M.A., Badran, A.H., et al. (2021) Clinically rele-
vant mutations in core metabolic genes confer antibiotic
resistance. Science 371: eaba0862.
Maeda, T., Sanchez-Torres, V., and Wood, T.K. (2008)
Metabolic engineering to enhance bacterial hydrogen pro-
duction. Microb Biotechnol 1: 30–39.
Manoharan, R.K., Mahalingam, S., Gangadaran, P., and
Ahn, Y.-H. (2020) Antibacterial and photocatalytic activi-
ties of 5-nitroindole capped bimetal nanoparticles
against multidrug resistant bacteria. Colloids Surf, B
188: 110825.
Marimon, O., Teixeira, J.M.C., Cordeiro, T.N., Soo, V.W.C.,
Wood, T.L., Mayzel, M., et al. (2016) An oxygen-sensitive
toxin–antitoxin system. Nat Commun 7: 13634.
McNulty, R., Sritharan, D., Liu, S., Hormoz, S., and Rosen-
thal, A. Z. (2021) Droplet-based single cell RNA sequenc-
ing of bacteria identifies known and previously unseen
cellular states. bioRxiv. 2021.2003.2010.434688.
Megaw, J., and Gilmore, B.F. (2017) Archaeal persisters:
persister cell formation as a stress response in Haloferax
volcanii. Front Microbiol 8: 1589.
Merfa, M.V., Niza, B., Takita, M.A., and De Souza, A.A.
(2016) The MqsR/A toxin-antitoxin system from Xylella
fastidiosa plays a key role in bacterial fitness, pathogenic-
ity, and persister cell formation. Front Microbiol 7: 904.
Ni, S., Li, B., Tang, K., Yao, J., Wood, T.K., Wang, P., and
Wang, X. (2021) Conjugal plasmid-encoded toxin–antitox-
in system PptT/PrpA directly controls plasmid copy
number. Proc Natl Acad Sci USA 118: e2011577118.
Ogura, T., and Hiraga, S. (1983) Mini-F plasmid genes that
couple host cell division to plasmid proliferation. Proc Natl
Acad Sci USA 80: 4784–4788.
Pecota, D.C., and Wood, T.K. (1996) Exclusion of T4 Phage
by the hok/sok Killer Locus from Plasmid R1. J Bacteriol
178: 2044–2050.
Powell, D.N., Swimm, A., Sonowal, R., Bretin, A., Gewirtz,
A.T., Jones, R.M., and Kalman, D. (2020) Indoles from the
commensal microbiota act via the AHR and IL-10 to
tune the cellular composition of the colonic epithelium
during aging. Proc Natl Acad Sci USA 117: 21519–21526.
Ren, D., Bedzyk, L.A., Thomas, S.M., Ye, R.W., and Wood,
T.K. (2004) Gene expression in Escherichia coli biofilms.
Appl Microbiol Biotechnol 64: 515–524.
Santi, I., Manfredi, P., Maffei, E., Egli, A., and Jenal, U.
(2021) Evolution of antibiotic tolerance shapes resistance
development in chronic Pseudomonas aeruginosa infec-
tions. mbio 12: e03482–e3420.
Favaro, M.T.d.P., Munar, D.M.M., Souza, A.A.d., Cotta,
M.A., and Souza, A.P.d. (2016) The antitoxin protein of
a toxin-antitoxin system from Xylella fastidiosa is
secreted via outer membrane vesicles. Front Microbiol
7: 2030.
Shimada, Y., Kinositha, M., Harada, K., Mizutani, M., Masah-
ata, K., Kayama, H., and Takeda, K. (2013) Commensal
bacteria-dependent indole production enhances epithelial
barrier function in the colon. PLoS One 8: e80604.
Song, S., Gong, T., Yamasaki, R., Kim, J.-S., and Wood,
T.K. (2019) Identification of a potent indigoid persister
antimicrobial by screening dormant cells. Biotechnol Bio-
engr 116: 2263–2274.
Song, S., and Wood, T.K. (2020) ppGpp ribosome dimeriza-
tion model for bacterial persister formation and resuscita-
tion. Biochem Biophys Res Com 523: 281–286.
Soutourina, O. (2019) Type I toxin-antitoxin systems in clos-
tridia. Toxins 11: 253.
Spichak, S., Bastiaanssen, T.F.S., Berding, K., Vickova, K.,
Clarke, G., Dinan, T.G., and Cryan, J.F. (2021) Mining
microbes for mental health: determining the role of micro-
bial metabolic pathways in human brain health and dis-
ease. Neurosci Biobehav Rev.
Sun, C., Guo, Y., Tang, K., Wen, Z., Li, B., Zeng, Z., and
Wang, X. (2017) MqsR/MqsA toxin/antitoxin system regu-
lates persistence and biofilm formation in Pseudomonas
putida KT2440. Front Microbiol 8: 840.
Sun, F., Bian, M., Li, Z., Lv, B., Gao, Y., Wang, Y., and Fu,
X. (2020) 5-Methylindole potentiates aminoglycoside
against gram-positive bacteria including Staphylococcus
aureus persister under hypoionic conditions. Front Cell
Infec Microbial 10: 84.
Thappeta, K.R.V., Vikhe, Y.S., Yong, A.M.H., Chan-Park,
M.B., and Kline, K.A. (2020) Combined efficacy of an
antimicrobial cationic peptide polymer with conventional
antibiotics to combat multidrug-resistant pathogens. ACS
Infec Dis 6: 1226–1237.
Wang, X., Kim, Y., Hong, S.H., Ma, Q., Brown, B.L., Pu, M.,
et al. (2011) Antitoxin MqsA helps mediate the bacterial
general stress response. Nat Chem Biol 7: 359–366.
Wang, X., Lord, D.M., Hong, S.H., Peti, W., Benedik, M.J.,
Page, R., and Wood, T.K. (2013) Type II toxin/antitoxin
MqsR/MqsA controls type V toxin/antitoxin GhoT/GhoS.
Environ Microbiol 15: 1734–1744.
Wang, X., Yao, J., Sun, Y.-C., and Wood, T.K. (2020) Type
II toxin/antitoxin classification system for antibiotics that
enzymatically neutralize toxins. Trends Microbiol.
Wang, Y., Zhang, S.-P., Zhang, M.-Y., Kempher, M.L., Guo,
D.-D., Han, J.-T., et al. (2019) The antitoxin MqsA homo-
logue in Pseudomonas fluorescens 2P24 has a rewired
regulatory circuit through evolution. Environ Microbiol
21: 1740–1756.
© 2021 The Author. Microbial Biotechnology published by Society for Applied Microbiology and John Wiley & Sons Ltd.
Wood, T.K., and Song, S. (2020) Forming and waking dormant cells: The ppGpp ribosome dimerization persister model. *Biofilm* 2: 100018.

Wozniak, R.A.F., and Waldor, M.K. (2009) A toxin-antitoxin system promotes the maintenance of an integrative conjugative element. *PLoS Genet* 5: e1000439.

Yam, Y.-K., Alvarez, N., Go, M.-L., and Dick, T. (2020) Extreme drug tolerance of *Mycobacterium abscessus* “Persisters”. *Front Microbiol* 11: 359.

Yamaguchi, Y., Park, J., and Inouye, M. (2011) Toxin-antitoxin systems in bacteria and archaea. *Annu Rev Genet* 45: 61–79.

Yamasaki, R., Song, S., Benedik, M.J., and Wood, T.K. (2020) Persister cells resuscitate using membrane sensors that activate chemotaxis. Lower cAMP levels, and revive ribosomes. *iScience* 23: 100792.

Zhang, W., Yamasaki, R., Song, S., and Wood, T.K. (2019) Interkingdom signal indole inhibits *Pseudomonas aeruginosa* persister cell waking. *J Appl Microbiol* 127: 1768–1775.