 doi:10.1111/j.1365-2958.2011.07922.x

MicroCommentary

# Bacterial L-forms on tap: an improved methodology to generate *Bacillus subtilis* L-forms heralds a new era of research

**Kevin M. Devine***
*Smurfit Institute of Genetics, Trinity College Dublin, Dublin 2, Ireland.*

## Summary

**Bacterial L-forms are cell wall-less forms of bacteria that usually grow with a conventional cell wall. Despite being important for research, L-forms are difficult to generate reproducibly and research in this area is challenging. The manuscript published in this issue by Domínguez-Cuevas and colleagues reports a method to rapidly, quantitatively and reproducibly generate populations of L-forms in *Bacillus subtilis*. Importantly, the methodology may be applicable to other bacteria heralding a new era of L-form research. Moreover, the genetic requirements of this method provide insights into how Lipid II synthesis and autolysin expression/activity are normally balanced and the central role of the WalRK two-component system in this process.**

'L-forms' are the wall-less form of a bacterium that usually grows with a conventional cell wall. They were first identified in *Streptobacillus moniliformis* by Klieneberger in 1935 and so named to honour the Lister Institute where she worked, but have since been reported for both Gram-positive and Gram-negative bacteria (for a review of their history see Domingue and Woody, 1997; Allan *et al*., 2009 and references therein). L-form research is important for several reasons: (i) the absence of a cell wall presents the opportunity to investigate its role in cellular processes such as cell division (Joseleau-Petit *et al*., 2007; Leaver *et al*., 2009), (ii) L-forms are clinically relevant since, lacking peptidoglycan, they are resistant to cell wall-acting antibiotics and do not trigger the innate immune response. Moreover, oscillation between L- and N-forms (normal form) may contribute to persistent bacterial infection (Domingue and Woody, 1997), (iii) studying L-forms may contribute to our understanding of the biology and evolution of the cell wall-less *Mollicute* class of bacteria and (iv) L-forms have been utilized in the biotechnology industry (Allan *et al*., 2009). However, research on L-forms is challenging, sometimes controversial and often lacking in reproducibility for reasons that emanate principally from the methods required to produce them. Generating L-forms from the parental N-form state requires serial passaging under conditions where cell wall synthesis is inhibited (by antibiotics) or the cell wall is degraded (by murolytic enzymes). Such L-forms are usually unstable during early passages, reverting to N-forms upon withdrawal of the conducive conditions. However, a stable L-form derivative is often achieved eventually and subsequent propagation requires only an osmoprotectant medium. Stable L-forms probably harbour multiple genetic mutations that reduce or prevent cell wall synthesis obviating the necessity for conducive environmental conditions. Perhaps controversy ought to be expected considering the plethora of conditions employed to generate unstable L-forms and the range of genetic mutations that might stabilize them.

In this issue Domínguez-Cuevas *et al*. (2011) make an important contribution to L-form research, reporting the development of a *Bacillus subtilis* strain (PDC134) that can be manipulated to quickly and quantitatively transition between a normal rod-shaped bacterium and a stable L-form. This development removes the variability heretofore inherent in L-form generation, heralding an era of research that can be conducted with strains of genetic integrity produced under standardized and reproducible conditions.

Two conditions are required to realize the N- to L-form transition, both of which are achieved genetically; peptidoglycan synthesis must be reduced in a strain with a defect either in cylindrical cell wall synthesis (*walRR204C*) or in septum formation (*sepFT11M*) (Domínguez-Cuevas *et al*., 2011). Peptidoglycan synthesis is controlled by

Accepted 8 November, 2011. *For correspondence. E-mail kdevine@tcd.ie; Tel. (+353) (01) 896 1872; Fax (+353) (01) 671 4968.

expressing the *murE* operon from a xylose-inducible promoter. Aberrant septum formation occurs when SepF is either mutated (T11M) or deleted. Aberrant cylindrical cell wall synthesis occurs when WalR is mutated (R204C) to generate a partially constitutive variant of the response regulator (Domínguez-Cuevas *et al*., 2011). WalRK (formerly named YycFG) is an essential two-component signal transduction system in *B. subtilis* that co-ordinates cylindrical cell wall synthesis with cell division (Fabret and Hoch, 1998; Bisicchia *et al*., 2007; Fukushima *et al*., 2008; 2011). In growing cells, a signal emanating from the divisome activates the WalK kinase which phosphoryates WalR~P to trigger autolysin (YocH, CwlO, LytE) expression, with concommitant repression of proteins (IseA, YjeA) that modulate autolysin activity. In non-dividing cells, autolysin expression is greatly reduced and autolysin activity is modulated or inhibited by increased IseA and YjeA levels (Bisicchia *et al*., 2007; Fukushima *et al*., 2008; 2011). Domínguez-Cuevas *et al*. (2011) show that when peptidoglycan synthesis is decreased, the partially constitutive WalR_R204C leads to continued autolysin expression (a condition under which their expression would normally be reduced) and a loss of cell wall integrity that permits escape of the L-form from the sacculus. A very satisfying aspect of their work is that L-forms escape from the sacculus at the location where the genetic lesion is manifest, beautifully shown in supplemental movies: escape occurs from the septum of cells carrying the *sepFT11M* mutation consistent with previous reports that SepF is required for normal septum formation (Hamoen *et al*., 2006); escape occurs from the cylinder of cells carrying the *walRR204C* mutation, consistent with the role of WalRK in synthesis of the cylindrical cell wall (Bisicchia *et al*., 2007). Moreover, L-forms are generated at a lower frequency and with delayed timing in cells with the septal defect when compared to cells with the cylindrical defect, while L-form generation is increased in strains with both mutations. The ability to generate L-forms synchronously and quantitatively in a time period of hours, rather than after serial passaging, and without addition of cell wall-acting agents, is a seminal technological development in this field. Future studies conducted with synchronous and homogeneous L-form populations of known genetic integrity should clarify many of the ambiguities that have arisen and should make them a useful and tractable system with which to address fundamental biological problems. The Errington group has now associated L-form generation in *B. subtilis* with mutation of three genes. The *walRR204C* and *sepFT11M* mutations promote L-form escape when combined with reduced peptidoglycan synthesis while mutation of *yqiD* (a homologue of *ispA* in *E. coli* that encodes a protein involved in the undecaprenol phosphate synthetic pathway) promotes stabilization and propagation of L-forms generated under conditions of reduced peptidoglycan synthesis (Leaver *et al*., 2009; Domínguez-Cuevas *et al*., 2011). It will be interesting to see if this combination of genetic manipulations, or at least application of the principles of these genetic manipulations, can generate L-forms in other bacteria, particularly in Gram-negative bacteria where cell envelope structure is very different to that of *B. subtilis*. Moreover, it will be interesting to determine whether mutation of these genes contributed to stabilization of previously established L-form lines.

The genetic modifications required to effect the N- to L-form transition suggest that cell wall integrity requires autolysin (YocH, CwlO, LytE) production and Lipid II (the peptidoglycan precursor) synthesis to be balanced. It is important to note that the *walRR204C* mutation alone does not lead to L-form escape (although small round osmotically sensitive cells are formed, similar to those previously reported by Fukuchi *et al*., 2000) despite elevated autolysin levels. Moreover, reducing Lipid II synthesis alone leads to cells with aberrant shapes that lyse unless stabilized by osmoprotectants and do not propagate (Leaver *et al*., 2009). However, the combination of elevated autolysin level coupled with reduced Lipid II synthesis results in a loss of cell wall integrity that allows protoplasts to escape from the resultant lesion in the sacculus. Domínguez-Cuevas *et al*. (2011) detail nuances in how balanced Lipid II synthesis and autolysin production is normally achieved by inhibiting cell wall metabolism with antibiotics that act at different steps of peptidoglycan synthesis. Inhibiting Lipid II synthesis with fosfomycin promotes L-form escape in strains carrying either *walRR204C* alone or both *walRR204C* and *sepFT11M* mutations but inhibiting incorporation of precursor into mature peptidoglycan with penicillin G does not. Future studies on the differential effects of these antibiotics may reveal how the kinetics of Lipid II production and utilization are normally balanced, and the role of WalRK in this processes.

Several features of this study extend our understanding of WalRK function in *B. subtilis*. The reason for WalRK essentiality in *B. subtilis* has not been established, and does not appear to result from controlling expression of a single essential gene as is the case for WalRK control of *pcsB* expression in *Streptococcus pneumoniae* (Ng *et al*., 2003, 2004; Bisicchia *et al*., 2007; Dubrac *et al*., 2008). However, the demonstration by Domínguez-Cuevas *et al*. (2011) that cell wall integrity requires Lipid II synthesis and autolysin production to be balanced may provide insight into WalRK essentiality. WalRK depletion in *B. subtilis* cells growing exponentially in LB medium (doubling time ~20 min) results in cell lysis. In such cells, Lipid II synthesis and autolysin (YocH, CwlO, LytE) production are imbalanced in a manner antithetical to that reported by Domínguez-Cuevas *et al*. (2011): in WalRK depleted

cells, autolysin production is greatly reduced under growth conditions that are conducive to a high level of Lipid II synthesis (Fabret and Hoch, 1998; Howell *et al*., 2003; Bisicchia *et al*., 2007). Thus cell lysis may result from a loss of cell wall integrity due this 'reversed' imbalance. It is interesting that WalRK depletion in cells growing at reduced growth rates (*ie* growth rates of ~ 50 min and > 200 min in LPDM and during phosphate limtiation respectively) does not lead to cell lysis (Howell *et al*., 2006; Bisicchia *et al*., 2010). Perhaps the reduced level of Lipid II synthesis under these growth conditions is insufficient to generate an imbalance of sufficient magnitude to compromise cell wall integrity. The concept of balanced autolysin and Lipid II synthesis may also explain the gene composition of the WalRK regulon and how each gene is regulated. WalRK controls autolysins (YocH, CwlO, LytE) and autolysin modulator (YjeA)/inhibitor (IseA) activities in opposite ways: when Lipid II synthesis is high (growing cells) autolysins are expressed and modulator/inhibitor activities are repressed; when Lipid II synthesis is low (non-growing cells) the situation is reversed with a low level of autolysin expression and a high level of expression of the autolysin modulator/inhibitor activities (Bisicchia *et al*., 2007; Salzberg and Helmann, 2007; Yamamoto *et al*., 2008). Thus it would appear that the lowered Lipid II synthesis level in non-growing cells requires not only that autolysin expression be reduced but also that the activity of autolysins already produced be inhibited. The increased IseA level is particularly significant since it inhibits D,L-endopeptidase (LytE, LytF, CwlO, CwlS) activity *in vitro* with evidence that it inhibits LytE activity *in vivo,* an autolysin that has a role in cell elongation (Carballido-López *et al*., 2006; Yamamoto *et al*., 2008). Together these observations suggest that the WalRK two-component system co-ordinates autolysin synthesis and activity with Lipid II synthesis to achieve the balance that is required for cell wall integrity.

The genetic analysis of WalRK function has been significantly hampered by its essentiality. Studies to date have utilized conditional WalRK expression (Howell *et al*., 2003; Ng *et al*., 2003; Dubrac and Msadek, 2004), a hybrid (PhoP'-'WalR) response regulator (Howell *et al*., 2003) and the partially constitutive WalR_D54H variant that results in a significant growth defect (Fukuchi *et al*., 2000). Thus the *walRR204C* partial gain of function allele reported by Domínguez-Cuevas *et al*. (2011) is a new and very useful genetic tool. The mutation (WalR_R204C) is located in the DNA recognition helix that makes WalR activity partially constitutive and independent of phosphorylation. The highly conserved nature of WalRK and the near invariance of the WalR DNA recognition helix in the *Bacillales*, *Lactobacillales* and some members of the *Clostridia* suggests that WalR_R204C variants in other bacteria may have similar partial gain of function activity, thereby greatly facilitating genetic analysis of this two-component system. Because its activity is phosphorylation independent, WalR_R204C variants will be especially useful is establishing the signals to which WalK responds. Shah and Dworkin (2010) show that YocH expression is responsive to muropeptides from exponentially growing cells in a manner that is dependent on the Ser/Thr protein kinase PrkC, suggesting that WalRK activation might be subject to additional control by this kinase. The WalR_R204 variant will help to discriminate the relative contributions of the WalK and PrkC kinases to WalR activation and establish how these signals are integrated to confer the unique promoter activity profiles displayed by WalRK regulon genes in *B. subtilis* (Botella *et al*., 2011).

In summary, the paper by Domínguez-Cuevas and colleagues makes several important contributions. They report a method to rapidly, quantitatively and reproducibly generate bacterial L-forms in *B. subtilis* heralding a new era of research in this area. Moreover the principles of this methodology may be widely applicable to other bacteria. Their results suggest that cell wall integrity requires Lipid II synthesis and autolysin expression/activity to be balanced and that the WalRK two-component system plays a crucial role in co-ordinating these activities under different growth conditions. The finding that the WalR_R204C mutation resides in a highly conserved region of the DNA recognition helix that makes its activity independent of phosphorylation will greatly facilitate genetic analysis of WalK kinase function and the signals to which it responds, not only in *B. subtlis*, but in a wide range of bacteria.

## Acknowledgements

Research in the Devine laboratory is supported by Science Foundation Ireland.

## References

Allan, E.J., Hoischen, C., and Gumpert, J. (2009) Bacterial L-forms. *Adv Appl Microbiol* **68:** 1–39.

Bisicchia, P., Noone, D., Lioliou, E., Howell, A., Quigley, S., Jensen, T., *et al*. (2007) The essential YycFG two-component system controls cell wall metabolism in *Bacillus subtilis*. *Mol Microbiol* **65:** 180–200.

Bisicchia, P., Lioliou, E., Noone, D., Salzberg, L.I., Botella, E., Hübner, S., *et al*. (2010) Peptidoglycan metabolism is controlled by the WalRK (YycFG) and PhoPR two-component systems in phosphate-limited *Bacillus subtilis* cells. *Mol Microbiol* **75:** 972–989.

Botella, E., Hübner, S., Hokamp, K., Hansen, A., Bisicchia, P., Noone, D., *et al*. (2011) Cell envelope gene expression in phosphate-limited *Bacillus subtilis* cells. *Microbiology* **157:** 2470–2484.

Carballido-López, R., Formstone, A., Li, Y., Ehrlich, S.D., Noirot, P., and Errington, J. (2006) Actin homolog MreBH

governs cell morphogenesis by localization of the cell wall hydrolase LytE. *Dev Cell* **11:** 399–409.

Domingue, G.J., and Woody, H.B. (1997) Bacterial persistence and expression of disease. *Clin Microbiol Rev* **10:** 320–344.

Domínguez-Cuevas, P., Mercier, R., Leavera, M., Kawai, Y., and Errington, J. (2011) The rod to L-form transition of *Bacillus subtilis* is limited by a requirement for the protoplast to escape from the cell wall sacculus. *Mol Microbiol* (in press).

Dubrac, S., and Msadek, T. (2004) Identification of genes controlled by the essential YycF/YycG two-component system of *Staphylococcus aureus*. *J Bacteriol* **186:** 1175–1181.

Dubrac, S., Bisicchia, P., Devine, K.M., and Msadek, T. (2008) A matter of life and death: cell wall homeostasis and the WalKR (YycGF) essential signal transduction pathway. *Mol Microbiol* **70:** 1307–1322.

Fabret, C., and Hoch, J.A. (1998) A two-component signal transduction system essential for growth of *Bacillus subtilis*: implications for anti-infective therapy. *J Bacteriol* **180:** 6375–6383.

Fukuchi, K., Kasahara, Y., Asai, K., Kobayashi, K., Moriya, S., and Ogasawara, N. (2000) The essential two-component regulatory system encoded by *yycF* and *yycG* modulates expression of the *ftsAZ* operon in *Bacillus subtilis*. *Microbiology* **146:** 1573–1583.

Fukushima, T., Szurmant, H., Kim, E.J., Perego, M., and Hoch, J.A. (2008) A sensor histidine kinase co-ordinates cell wall architecture with cell division in *Bacillus subtilis*. *Mol Microbiol* **69:** 621–632.

Fukushima, T., Furihata, I., Emmins, R., Daniel, R.A., Hoch, J.A., and Szurmant, H. (2011) Role for the essential YycG sensor histidine kinase in sensing cell division. *Mol Microbiol* **79:** 503–522.

Hamoen, L.W., Meile, J.C., de Jong, W., Noirot, P., and Errington, J. (2006) SepF, a novel FtsZ-interacting protein required for a late step in cell division. *Mol Microbiol* **59:** 989–999.

Howell, A., Dubrac, S., Andersen, K.K., Noone, D., Fert, J., Msadek, T., *et al*. (2003) Genes controlled by the essential YycG/YycF two-component system of *Bacillus subtilis* revealed through a novel hybrid regulator approach. *Mol Microbiol* **49:** 1639–1655.

Howell, A., Dubrac, S., Noone, D., Varughese, K.I., and Devine, K.M. (2006) Interactions between the YycFG and PhoPR two-component systems in *Bacillus subtilis*: the PhoR kinase phosphorylates the non-cognate YycF response regulator upon phosphate limitation. *Mol Microbiol* **59:** 1199–1215.

Joseleau-Petit, D., Liebart, J.C., Ayala, J.A., and D'Ari, R. (2007) Unstable *Escherichia coli* L-forms revisited: growth requires peptidoglycan synthesis. *J Bacteriol* **189:** 6512–6520.

Leaver, M., Domínguez-Cuevas, P., Coxhead, J.M., Daniel, R.A., and Errington, J. (2009) Life without a wall or division machine in *Bacillus subtilis*. *Nature* **457:** 849–853.

Ng, W.L., Robertson, G.T., Kazmierczak, K.M., Zhao, J., Gilmour, R., and Winkler, M.E. (2003) Constitutive expression of PcsB suppresses the requirement for the essential VicR (YycF) response regulator in *Streptococcus pneumoniae* R6. *Mol Microbiol* **50:** 1647–1663.

Ng, W.L., Kazmierczak, K.M., and Winkler, M.E. (2004) Defective cell wall synthesis in *Streptococcus pneumoniae* R6 depleted for the essential PcsB putative murein hydrolase or the VicR (YycF) response regulator. *Mol Microbiol* **53:** 1161–1175.

Salzberg, L.I., and Helmann, J.D. (2007) An antibiotic-inducible cell wall-associated protein that protects *Bacillus subtilis* from autolysis. *J Bacteriol* **189:** 4671–4680.

Shah, I.M., and Dworkin, J. (2010) Induction and regulation of a secreted peptidoglycan hydrolase by a membrane Ser/Thr kinase that detects muropeptides. *Mol Microbiol* **75:** 1232–1243.

Yamamoto, H., Hashimoto, M., Higashitsuji, Y., Harada, H., Hariyama, N., Takahashi, L., *et al*. (2008) Post-translational control of vegetative cell separation enzymes through a direct interaction with specific inhibitor IseA in *Bacillus subtilis*. *Mol Microbiol* **70:** 168–182.