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The Editors hope that readers will take full advantage of this section and use it to raise matters that hitherto have been confined to a limited audience.

***Christopher M. Thomas*, Editor-in-chief**

Models on stickiness of replicated *Escherichia coli oriC*

In a recent paper Hiraga and coworkers (10) presented a model on *Escherichia coli* DNA segregation in which duplicated *oriC* regions remain stuck together during a large part (about 60 %) of the DNA replication cycle. In a *dnaC* mutant this phenomenon is even more pronounced: separation of *oriC* regions more or less coincides with termination of DNA replication. In an explanatory model (Fig. 7 in ref. 10) the authors explicitly compare the interpretation of their data (model 1; cohesion model) with *their* interpretation of the data of Roos *et al.* (8) (model 2; immediate separation model). We wish to comment on this comparison.

For any reliable interpretation of the sequential separation of gene regions, the temporal relationship between the DNA replication period and the cell division cycle should be known. It tells which genes (labelling targets) have duplicated at a particular time or cell length. For instance, a newborn *E. coli* K-12 cell does not need to start with DNA replication. Under many experimental conditions as specified below DNA replication is already halfway when a cell is born (Fig. 1a).

In their Fig. 7 Sunako *et al.* (10) depict DNA replication cycles of cells growing with doubling times of 80, 60 and 36 min at 37 °C (Fig. 7a, b and c, respectively). They assumed a replication time period C of 40 min and a division time period D (the time between termination of DNA replication and division) of 20 min under those growth conditions. The above cell cycle periods are based on the classic experiments of Helmstetter & Cooper (4). However, those experiments had been done with *E. coli* B/r grown at 37 °C, whereas here we are concerned with *E. coli* K-12 strains growing at either 28 or 37 °C.

In a careful study Bipatnath *et al.* (1) determined a C period of 58 min for *E. coli* K-12 (strain AB1157) growing at 37 °C with a doubling time of 54 min. This C value is comparable to the value of 53 min which we have determined for a K-12 culture (strain MC4100) growing in glucose minimal medium at 37 °C with a doubling time of 55 min (Woldringh *et al.*, unpublished data). Taking into account the duration of the D period, newborn K-12 cells already contain two origins (Fig. 1a). In fact this can also be derived from the flow-cytometric data of Hiraga *et al.* (Fig. 3A in ref. 5). Their data on cellular DNA content (85 % of cells contain two origins) of a culture growing with a doubling time of 55 min at 37 °C are thus at odds with the DNA replication scheme of cells with a doubling time of 60 min as depicted in Fig. 7B of Sunako *et al.* (10). Making their comparison with the data of Roos *et al.* (8) they state that our cells were 'growing randomly in a rich medium' with a doubling time of 36 min (cf. Fig. 7C in ref. 10). On the contrary, in fact the cells were grown in glucose minimal medium at 28 °C with a doubling time of 80 min (8) and a C period of 70 min (6). Clearly, care should be exerted when comparing DNA replication periods with respect to strains, growth medium and temperature as well as published work.

What about the interpretation of FISH (fluorescence *in situ* hybridization) data? We will focus the discussion on the localization of *oriC* by FISH. As documented extensively in our work (8), newborn *E. coli* K-12 cells grown with a doubling time of about 80 min at 28 °C have replicated about 50 % of their DNA and thus contain two *ori*Cs. In small cells often one focus was seen asymmetrically located in the cell (Fig. 1b), whereas in the case of two foci they were positioned diametrically opposite to each other (Fig. 1c). Thus, cells of the same length class can have either one *oriC* focus asymmetrically located in the cell or two foci, one in each cell half (5, 7, 8). This has been interpreted to mean that upon duplication one origin moves speedily to the other cell half (Fig. 1d; 7). In contrast, we have argued that lack of foci is due to limited labelling efficiency of the FISH procedure (8). If the cohesion model would apply, this would mean that because single foci have an asymmetric position in the cell, the replication machinery is likewise asymmetrically positioned (7) and not in the cell centre as stated most recently by Sunako *et al.* (10). The supposed cohesion has another consequence. Provided that the replicating nucleoid as a whole separates in line with cell elongation, while keeping a constant distance to each cell pole (11), it follows that gene regions other than the origin and its surroundings should occupy cellular positions away from the sticky origins. This requires further assessment.

To further address the question of the initial separation of DNA regions 'we simulated FISH experiments by calculating the theoretical intracellular positions of *oriC* for

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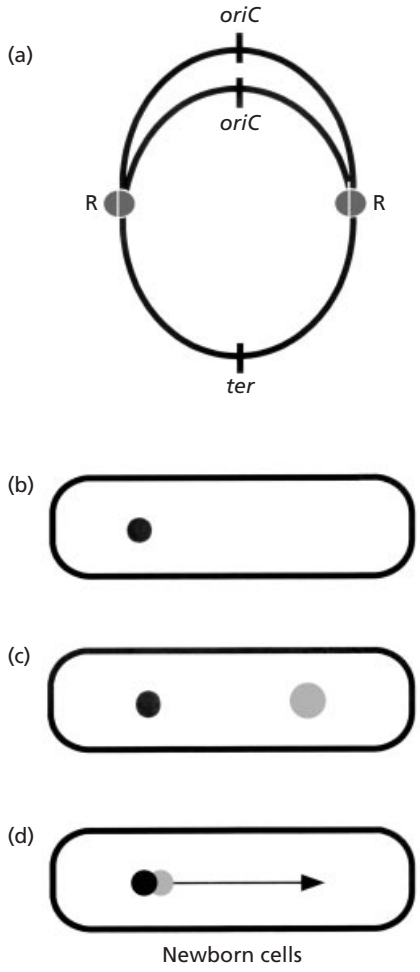

Fig. 1. DNA replication pattern and the localization of fluorescent foci in a newborn K-12 cell (see also accompanying text). (a) Typically at moderately slow growth (doubling times of about 55 to 85 min at 28 or 37 °C) the bacterial chromosome is about 50 per cent replicated in a newborn cell. *oriC*, origin of replication; *ter*, terminus; R, replication apparatus. (b) A newborn cell with only one of the two *ori*Cs labelled (see ref. 8). (c) A newborn cell with two *ori*Cs labelled. (d) One of the *oriCs* duplicated at the cell pole moves speedily to the opposite pole (see ref. 7).

a population of cells and incorporating the following factors: measurement error, resolution of fluorescence microscopy, the probability that an *oriC* region is labelled and a normally distributed variation in the position of *oriC* regions' (9). We have tested two models (Fig. 2 in ref. 9). One model was the same as the immediate separation model. The other resembled closely the cohesion model, that is, the duplicated origins stick together for 50% of the DNA replication period and duplication takes place at a prospective cell centre (7). We have concluded: 'The simulated data of these models are very similar, indicating that FISH is not a suitable method to distinguish between these two models' (9). Even if FISH were perfect (with respect to labelling efficiency and cellular localization of the probe), one should not ignore the resolution limit of the light microscope (about 250 nm; cf. ref. 9). The cohesion and immediate separation models [models 1 and 2 in Fig. 7 of Sunako *et al.* (10)], would resemble each other closely if the practical factors referred to above had been incorporated.

What about separation of *oriC* regions in living cells when they have been labelled with a fusion protein containing green fluorescent protein (GFP)? It has been pointed out that duplicated foci need a certain distance before they become visible as two (2, 8) and that they move apart bidirectionally at a speed that exceeds cell elongation (2). In principle this leaves room for 'cohesion' of the origins for a short period. However, cell growth (cell elongation) can be slowed down in GFP fusion protein-containing cells (3). If this is not so for DNA replication and if 'DNA synthesis may provide the motive force to push subsequently replicated DNA towards the origins' (2) one can envisage a discrepancy between the velocity of cell elongation and the velocity of *oriC* separation. Even so, this does not answer the question of stickiness. Presumably, a more definite answer could be obtained if it were possible to label origins in living cells in such a way that cells can be grown under steady state conditions.

Nanne Nanninga, Marco Roos and Conrad L. Woldringh

Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands

Author for correspondence: Nanne Nanninga. Tel: +31 20 525 5194. Fax: +31 20 525 6271. e-mail: nanninga@science.uva.nl

1. Bipatnath, M., Dennis, P. P. & Bremer, H. (1998). Initiation and velocity of chromosome replication in *Escherichia coli* B/r and K-12. *J Bacteriol* **180**, 265–273.
2. Gordon, G. S. & Wright, A. (2000). DNA segregation in bacteria. *Annu Rev Microbiol* **54**, 681–708.
3. Gordon, G. S., Sitnikov, D., Webb, C. D., Teleman, A., Straight, A, Losick, R., Murray, A. W. & Wright, A. (1997). *Cell* **90**, 1113–1121.
4. Helmstetter, C. E. & Cooper, S (1968). DNA synthesis during the division cycle of rapidly growing *E. coli* B/r. *J Mol Biol* **31**, 507–518.
5. Hiraga, S., Ichinose, C., Niki, H. & Yamazoe, M. (1998). Cell cycle-dependent duplication and bidirectional migration of SeqA-associated DNA-protein complexes in *E. coli*. *Mol Cell* **1**, 381–387.
6. Huls, P., Vischer, N. O. E. & Woldringh, C. L. (1999). Delayed nucleoid segregation in *Escherichia coli*. *Mol Microbiol* **33**, 959–970.
7. Niki, H. & Hiraga, S. (1998). Polar localization of the replication origin and terminus in *Escherichia coli* nucleoids during chromosome partitioning. *Genes Dev* **12**, 1036–1045.
8. Roos, M., van Geel, A., Aarsman, M., Veuskens, J., Woldringh, C. L. & Nanninga, N. (2001a). The replicated *ftsQAZ* and *minB* chromosomal regions of *Escherichia coli* segregate on average in line with nucleoid movement. *Mol Microbiol* **39**, 633–640.
9. Roos, M., Lingeman, R., Woldringh, C. L. & Nanninga, N. (2001b). Experiments on movement of DNA regions in *Escherichia coli* evaluated by computer simulation. *Biochimie* **83**, 67–74.
10. Sunako, Y., Onogi, T. & Hiraga, S. (2001). Sister chromosome cohesion of *Escherichia coli*. *Mol Microbiol* **42**, 1233–1241.
11. van Helvoort, J. M. L. M., & Woldringh, C. L. (1994). Nucleoid partitioning in *Escherichia coli* during steady state growth and upon recovery from chloramphenicol treatment. *Mol Microbiol* **13**, 577–583.

A reply to this article will be published in *Microbiology* **148**, part 12.

Active expression of soluble methane monooxygenase from *Methylosinus trichosporium* OB3b in heterologous hosts

The soluble methane monooxygenase enzymes (sMMO) from the methanotrophs *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Bath) have been intensely studied since they hold promise for many applications including the reduction of the greenhouse gas methane (5) and the aerobic degradation of recalcitrant compounds [the US Environmental Protection Agency priority pollutant trichloroethylene is degraded 50 times faster by sMMO (1, 13)]. Similar monooxygenases from this class of bis-μ-hydroxo-bridged binuclear iron cluster proteins have also been evolved using DNA shuffling for the oxidation of compounds such as naphthalene to 1-naphthol with the goal of making synthetic chemicals while generating less waste (2). Hence, sMMO and related monooxygenases have been intensely studied both in terms of their kinetics (5, 16) as well as structure (4, 14, 17).

Unfortunately, there has been some confusion in the literature about expression of active sMMO. Recently, researchers from the University of Warwick have published two papers in which our expression of sMMO in 1994 was questioned. In these reports it was mistakenly reported that sMMO had little or no activity in *Pseudomonas putida* F1 (9) as well as that active expression of sMMO had been unsuccessful due to inactive hydroxylase in heterologous hosts (10). These reports were in error in that our group published two much earlier reports of the successful expression of active recombinant sMMO (6, 8).

We achieved (for the first time) active expression of sMMO from *mmoXYBZYC* of *M. trichosporium* OB3b in *Pseudomonas putida* F1 (6). The pollutant trichloroethylene was degraded at 5 nmol min^{-1} (mg protein)$^{-1}$ using whole cells and stoichiometric amounts of chloride were generated to show active expression of this large enzyme. Further evidence that sMMO was expressed in active form in *P. putida* F1 was obtained from our results that *P. putida* F1 without the *mmo*

locus was unable to degrade TCE in 43 different sets of experiments when this strain was grown with glucose in the absence of toluene, conditions that do not allow for expression of toluene dioxygenase (6); hence, the ability to degrade TCE was from the expression of active sMMO in this strain. Further, *P. putida* F1 is unable to degrade chloroform even under conditions that express its toluene dioxygenase (3, 6, 11) but when the *mmo* genes were expressed in this host, the recombinant strain degraded chloroform as a result of active expression of sMMO as shown by disappearance of the original compound and stoichiometric accumulation of the final product chloride (6). Therefore, active sMMO was shown in whole cells using trichloroethylene and chloroform assays, and copper repression of the *mmo* locus was eliminated cloning under the control of the *tac* promoter.

sMMO activity was also shown to occur in whole cells of *Agrobacterium tumefaciens* A114 and in *Rhizobium meliloti* 102F34 so the existence of at least three different non-methanotroph hosts with active sMMO expression were shown by 1996 (8). In this second report, crude cell extracts were also shown to have measurable *in vitro* sMMO activity using a propene assay (although comparisons of the crude extracts of the recombinant strain with purified sMMO components makes direct comparisons unreliable). In addition, increasing the dilution rate of chemostats increased sMMO activity in the recombinant strains (8). It is regrettable that it was reported in 1999 that there was only negligible sMMO activity as evidenced by the propene assay (10). Therefore, the report by University of Warwick group in 1999 was really the third report of expression of active sMMO. In both our initial reports, it was made clear that the sMMO activity was not as robust as that of sMMO activity in the methanotrophic host. However, the active expression was conclusive and reproducible.

It was also published in 1996 that copper ions affect both the reductase as well as the hydroxylase activity (7). So copper affects the expression of sMMO in methanotrophs by inhibiting transcription (12) as well as by inactivating existing sMMO.

To date, no one has achieved expression of active sMMO (i.e., active hydroxylase subunit) in *Escherichia coli* and even expression in methanotrophs has been problematic (9). However, there is compelling evidence that this enzyme should be able to be expressed in *E. coli* (perhaps if the hydroxylase is mutated since the other components are active). Beyond the published expression of active sMMO in *Agrobacterium*, *Rhizobium* and *Pseudomonas* strains, there are numerous reports of robust expression in *E. coli* of similar monooxygenases that are also expressed from six genes and contain bis-μ-hydroxo-bridged binuclear iron clusters (2, 15, 18). Therefore, it does not appear that a necessary gene is missing that is responsible for sMMO activity in *E. coli*. Perhaps directed evolution will lead to even more robust sMMO activity and breakthroughs in the understanding of how this particular enzyme efficiently cleaves the carbon-hydrogen bond of methane.

Thomas K. Wood

Departments of Chemical Engineering and Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269-3222, USA.

Tel: +1 860 486 2483. Fax: +1 860 486 2959.
e-mail: twood@engr.uconn.edu

1. Brusseau, G. A., Tsien, H.-C., Hanson, R. S. & Wackett, L. P. (1990). Optimization of trichloroethylene oxidation by methanotrophs and the use of a colorimetric assay to detect soluble methane monooxygenase activity. *Biodegradation* **1**, 19–29.
2. Canada, K. A., Iwashita, S., Shim, H. & Wood, T. K. (2002). Directed evolution of toluene *ortho*-monooxygenase for enhanced 1-naphthol synthesis and chlorinated ethene degradation. *J Bacteriol* **184**, 344–349.
3. Chauhan, S., Barbieri, P. & Wood, T. K. (1998) Oxidation of trichloroethylene, 1,1-dichloroethylene, and chloroform by toluene/*o*-xylene monooxygenase from *Pseudomonas stutzeri* OX1. *Appl Environ Microbiol* **64**, 3023–3024.
4. Elango, N., Radhakrishnan, R., Froland, W. A., Wallar, B. J., Earhart, C. A., Lipscomb, J. D. & Ohlendorf, D. H. (1997). Crystal structure of the hydroxylase component of methane monooxygenase from *Methylosinus trichosporium* OB3b. *Protein Sci* **6**, 556–568.
5. Gallagher, S. C., Callaghan, A. J., Zhao, J., Dalton, H. & Trewhella, J. (1999). Global conformational changes control the reactivity of methane monooxygenase. *Biochemistry* **38**, 6752–6760.
6. Jahng, D. & Wood, T. K. (1994). Trichloroethylene and chloroform degradation by a recombinant pseudomonad expressing soluble methane monooxygenase from *Methylosinus trichosporium* OB3b. *Appl Environ Microbiol* **60**, 2473–2482.
7. Jahng, D. & Wood, T. K. (1996). Metal ions and chloramphenicol inhibition of soluble methane monooxygenase from *Methylosinus trichosporium* OB3b. *Appl Microbiol Biotechnol* **45**, 744–749.
8. Jahng, D., Kim, C. S., Hanson, R. S. & Wood, T. K. (1996). Optimization of trichloroethylene degradation using soluble methane monooxygenase of *Methylosinus trichosporium* OB3b expressed in recombinant bacteria. *Biotechnol Bioeng* **51**, 349–359.
9. Lloyd, J. S., deMarco, P., Dalton, H. & Murrell, J. C. (1999). Heterologous expression of soluble methane monooxygenase genes in methanotrophs containing only particulate methane monooxygenase. *Arch Microbiol* **171**, 364–370.
10. Lloyd, J. S., Finch, R., Dalton, H. & Murrell, J. C. (1999). Homologous expression of soluble methane monooxygenase genes in *Methylosinus trichosporium* OB3b. *Microbiology* **145**, 461–470.
11. McClay, K., Fox, B. G. & Steffan, R. J. (1996). Chloroform mineralization by toluene-oxidizing bacteria. *Appl Environ Microbiol* **62**, 2716–2722.
12. Nielsen, A. K., Gerdes, K. & Murrell, J. C. (1997). Copper-dependent reciprocal transcriptional regulation of methane monooxygenase genes in *Methylococcus capsulatus* and *Methylosinus trichosporium*. *Mol Microbiol* **25**, 399–409.
13. Oldenhuis, R., Oedzes, J. Y., Waarde, J. J. V. D. & Janssen, D. B. (1991). Kinetics of chlorinated hydrocarbon degradation by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene. *Appl Environ Microbiol* **57**, 7–14.
14. Rosenzweig, A. C., Frederick, C. A., Lippard, S. J. & Nordlund, P. (1993). Crystal structure of a bacterial non-haem iron hydroxylase that catalyses the biological oxidation of methane. *Nature* **366**, 537–543.
15. Ryoo, D., Shim, H., Canada, K., Barbieri, P. & Wood, T. K. (2000). Aerobic degradation of tetrachloroethylene by toluene-*o*-xylene monooxygenase of *Pseudomonas stutzeri* OX1. *Nat Biotechnol* **18**, 775–778.
16. Wallar, B. J. & Lipscomb, J. D. (2001). Methane monooxygenase component b mutants alter the kinetics of steps throughout the catalytic cycle. *Biochemistry* **40**, 2220–2233.
17. Walters, K. J., Gassner, G. T., Lippard, S. J. & Wager, G. (1999). Structure of the soluble methane monooxygenase regulatory protein B. *Proc Natl Acad Sci USA* **96**, 7877–7882.
18. Winter, R. B., Yen, K.-M. & Ensley, B. D. (1989). Efficient degradation of trichloroethylene by a recombinant *Escherichia coli*. *Bio/Technology* **7**, 282–285.

Expression of soluble methane monooxygenase genes

In a paper entitled 'Homologous expression of soluble methane monooxygenase genes in *Methylosinus trichosporium* OB3b' (3) we failed to mention two papers which had, prior to that publication, reported for the first time the expression of the genes encoding the soluble methane monooxygenase (sMMO) gene cluster in *Pseudomonas putida* and other Gram negative bacteria. In the first paper (1) the sMMO gene cluster from *Methylosinus trichosporium* OB3b was expressed on a broad host range plasmid in *P. putida* and trichloroethylene (TCE) degradation rates [$V_{max} = 5$ nmol min^{-1} (mg protein)$^{-1}$] by the recombinant strain were reported. The second paper (2) described the optimization of sMMO activity in *P. putida* and expression of sMMO genes in *Rhizobium meliloti* and *Agrobacterium tumefaciens*. Under optimum conditions TCE degradation by a recombinant *P. putida* containing sMMO genes was 6–8 μM TCE degraded in 5 h using 5 ml resting cells of an $OD_{600} = 2$ ($\sim$ 2·5 mg dry cell wt).

The Lloyd *et al.* paper (3) described expression of sMMO genes from *M. trichosporium* OB3b in an sMMO-deletion strain of *M. trichosporium*. This system for the homologous expression of sMMO genes yielded cell-free extracts with specific activities of 140 nmol min^{-1} (mg protein)$^{-1}$ for sMMO when assayed using the conversion of propylene to propylene oxide (a standard sMMO assay used in many laboratories). This specific activity was over threefold higher than observed in cell-free extracts of wild-type *M. trichosporium* OB3b. Because this activity was so much higher than observed in the heterologous system described by the Wood laboratory and because in fact the systems were so different, we were unsure whether it was appropriate to cite this prior work. We realize that this was a mistake.

Recent work in our laboratory has given more of an insight into why only relatively low activities of sMMO are obtained when

sMMO genes are expressed in heterologous hosts. We have recently identified a *groEL* gene located 5′ of the *mmoX* gene cluster in *M. trichosporium* OB3b which, when mutated, results in a mutant with an sMMO-minus phenotype (Murrell *et al*., unpublished observations). This *groEL*, which is not present on the sMMO gene cluster constructs used in experiments by Wood and colleagues (1, 2), may be essential for the correct assembly of the sMMO or sMMO regulatory proteins, which could account for the high level expression of sMMO in the homologous host since this *groEL* is present both on the chromosome of *M. trichosporium* OB3b and on the expression plasmid used (3).

J. Colin Murrell

Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, UK

Tel: +44 24 76523553. Fax: +44 24 76523568. e-mail: cmurrell@bio.warwick.ac.uk

1. Jahng, D. & Wood, T. K. (1994). Trichloroethylene and chloroform degradation by a recombinant pseudomonad expressing soluble methane monooxygenase from *Methylosinus trichosporium* OB3b. *Appl Environ Microbiol* **60**, 2473–2482.

2. Jahng, D., Kim, C. S., Hanson, R. S. & Wood, T. K. (1996). Optimization of trichloroethylene degradation using soluble methane monooxygenase of *Methylosinus trichosporium* OB3b expressed in recombinant bacteria. *Biotechnol Bioeng* **51**, 349–359.

3. Lloyd, J. S., Finch, R., Dalton, H. & Murrell, J. C. (1999). Homologous expression of soluble methane monooxygenase genes in *Methylosinus trichosporium* OB3b. *Microbiology* **145**, 461–470.

What does a bacterial genome sequence represent? Misassignment of MAFF 303099 to the genospecies *Mesorhizobium loti*

Doolittle (1) recently discussed the potential and limitations of bacterial genome sequencing, and emphasized the importance of lateral gene transfers in bacterial adaptation, e.g. to become pathogens. The transferable genes, or 'accessory genome', should comprise genes that are advantageous intermittently and are not uniformly distributed among individuals of a species, though they may be shared between species. The important phenotypes that they encode are often of economic interest to medicine, e.g. pathogenicity islands and antibiotic resistance, and agriculture, e.g. symbiotic nitrogen fixation by rhizobia in association with plant roots. Such traits are sometimes used to identify bacteria, but this can lead to an 'identity crisis'. This is illustrated by the largest genome that Doolittle (1) discusses, that of a strain of rhizobia (MAFF 303099) isolated from a *Lotus corniculatus* root nodule. The strain was identified as *Mesorhizobium loti* but we suggest, based on phylogeny, that it belongs to a different species: *Mesorhizobium huakuii* biovar *loti*. Host-range variants in rhizobia are generally called 'biovars', in which the chromosome type (basic genome) defines the species and the symbiosis type (accessory genome) the biovar. MAFF 303099 differs considerably from the *M. loti* type strain at four chromosomal loci (*rrn*, *glnA*, *glnII*, *recA*; Fig. 1) and more resembles the type strain of *M. huakuii* (every bacterial species is defined by similarity to a single representative, the 'type strain').

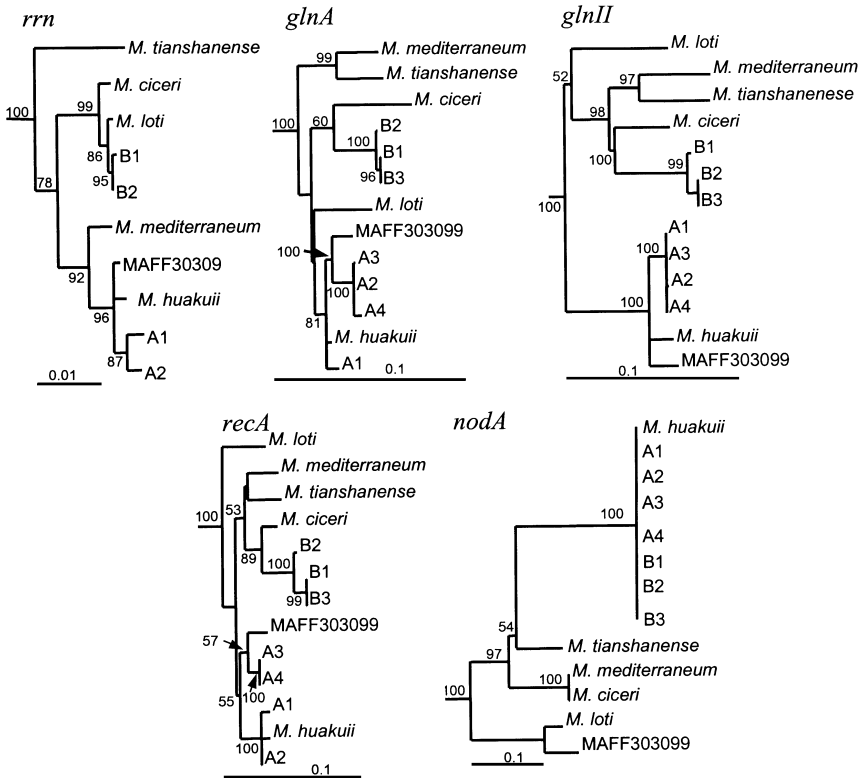

Fig. 1. Phylogenetic trees showing evolutionary relationships among *Mesorhizobium* strains for five partial gene sequences: 16S *rrn* (606–1420 bp, gaps ignored in pair-wise comparisons), *glnA* (936 bp), *glnII* (828 bp), *recA* (440 bp) and *nodA* (470 bp). Sequences labelled A1–4 (strains HN8A1, ZJ15B7, JX2B5 and JS5A15) and B1–3 (HB5A4, HN14A16 and HN15B23) are from isolates that nodulate *Astragalus sinicus* (7), MAFF 303099 indicates sequences taken from the published genome sequence. These sequences were amplified and sequenced according to the methods described in and compared to the corresponding sequences of *Mesorhizobium* type strains described in: ref. 2 for 16S *rrn* and *recA*; ref. 5 for *glnA* and *glnII* and ref. 7 for *nodA*. All trees were calculated using PAUP* (version 4.0b10; http://paup.csit.fsu.edu/) using the neighbour-joining algorithm with the HKY model. Trees were rooted using the corresponding *Sinorhizobium meliloti* sequence (not shown on trees). Bootstrap percentages shown on each branch were estimated from 1000 replicates. New sequences for the *A. sinicus* isolates have been deposited in the databases under accession numbers AJ459585–AJ459605.

The affiliation with *M. huakuii* is particularly striking for glutamine synthetase II (*glnII*), as MAFF 303099 shares a distinctive sequence thought to have been acquired by the ancestor of the *M. huakuii* lineage through lateral gene transfer from a *Rhizobium*-like species (5). We found this same *glnII* signature in four strains representing the dominant (> 96%) chromosomal type (6) among 204 isolates from *Astragalus sinicus*, the typical host of *M. huakuii* (type A1–4 in Fig. 1). Three strains representing the remaining genotypic diversity belong to a different lineage (type B1–3 in Fig. 1). We conclude that CCBAU 2609^T is truly representative of *M. huakuii*, and that MAFF 303099 belongs to this species.

In contrast, MAFF 303099 has symbiosis genes (represented here by *nodA*) that are most similar to those of other *Lotus* symbionts (Fig. 1). Symbiosis genes are mobile, being usually found on plasmids or transmissible genetic islands. All *A. sinicus* symbionts screened to date have identical *nodA* sequences (7), indicating relatively recent (in evolutionary time) transfer of their symbiosis genes between species. More directly, Sullivan *et al*. (4) detected transfer of the genes for nodulation of *Lotus* from an inoculant strain into several different chromosomal backgrounds within four years.

Lateral gene transfer thus shapes the bac-

terial genome on two different time scales. The transfer of *glnII* from *Rhizobium* to *Mesorhizobium* was apparently a single, rare event, since other basic 'housekeeping' genes generally share a consistent phylogeny (2, 5). By contrast, the accessory genome, here represented by symbiosis genes, undergoes detectable transfers within and between species. Accessory DNA makes up 10–25 % of the DNA in the three rhizobial genomes sequenced so far [*M. loti* (NC_002678), *Sinorhizobium meliloti* (NC_003047) and *Agrobacterium tumefaciens* (NC_003305)].

Sequencing an individual bacterial genome will provide a clear picture of the basic genome of the species, but only an arbitrary 'snapshot' of that part of the accessory gene pool that happens to be in the chosen isolate. When genome sequences are available from more than one strain of a species, e.g. *Escherichia coli*, we see clearly that they differ in their complement of accessory genes (3), so that a single strain does not adequately represent the whole species. Characterizing and sequencing more than one member of a species is therefore important, since this will define the common core of genes which defines the species. It will also allow identification of the associated accessory gene pool that provides a species with its adaptations to different niches and determines the variety of key properties such as symbioses or diseases with which the species is associated (1).

Sarah L. Turner,[1]† Xue-Xian Zhang,[2]† Fu-Di Li[2] and J. Peter W. Young[1]

[1]Department of Biology, University of York, PO Box 373, York YO10 5YW, UK.
[2]Department of Microbiology, Huazhong Agricultural University, China.
†Present address: CEH Oxford, Mansfield Road, Oxford OX1 3SR, UK.

Author for correspondence: Sarah L. Turner. Tel: +44 1865 281 630. Fax: +44 1865 281 696. e-mail: sltu@ceh.ac.uk

1. Doolittle, R. F. (2002). Microbial genomes multiply. *Nature* **416**, 697–700.
2. Gaunt, M. J., Turner, S. L., Rigottier-Gois, L., Lloyd-Macgilp, S. A. & Young, J. P. W. (2001). Phylogenies of *atpD* and *recA* support the SSU rRNA-based classification of rhizobia. *Int J Syst Evol Microbiol* **51**, 2037–2048.
3. Perna, N. T., Plunkett, G., Burland, V. & 25 other authors (2001). Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature* **409**, 529–533.
4. Sullivan, J. T., Eardly, D. B., van Berkum, P. & Ronson, C. W. (1996). Four unnamed species of nonsymbiotic rhizobia isolated from the rhizosphere of *Lotus corniculatus*. *Appl Environ Microbiol* **62**, 2818–2825.
5. Turner, S. L. & Young, J. P. W. (2000). The glutamine synthetases of rhizobia: phylogenetics and evolutionary implications. *Mol Biol Evol* **17**, 309–319.
6. Zhang, X. X., Guo, X. W., Terefework, Z., Cao, Y. Z., Hu, F. R, Lindstrom, K & Li, F. D. (1999). Genetic diversity among rhizobial isolates from field grown *Astragalus sinicus* of Southern China. *Syst Appl Microbiol* **22**, 312–320.
7. Zhang, X. X., Turner, S. L., Guo, X. W., Yang, H. J., Debellé, F., Yang, G. P., Dénarié, J., Young, J. P. W. & Li, F. D. (2000). The common nodulation genes of *Astragalus sinicus* rhizobia are conserved despite chromosomal diversity. *Appl Environ Microbiol* **66**, 2988–2995.

MprF-mediated lysinylation of phospholipids in *Bacillus subtilis* – protection against bacteriocins in terrestrial habitats?

A common strategy for organisms to inhibit bacteria is the production of antimicrobial peptides that damage bacterial membranes and that usually have cationic properties to enable efficient interactions with the anionic polymers in bacterial cell envelopes. Such cationic antimicrobial peptides (CAMPs) are produced by the innate immune systems of humans, animals and plants (e.g. defensins) (7) and many of the bacterial bacteriocins belong to the same class of molecules (3).

The recently described bacterial mechanisms protecting against a wide range of CAMPs may be beneficial in many kinds of environments. Most of these mechanisms involve modulations of the net charge in the bacterial cell envelope to reduce accumulation of cationic peptides (4). Modification of teichoic acids with positively charged D-

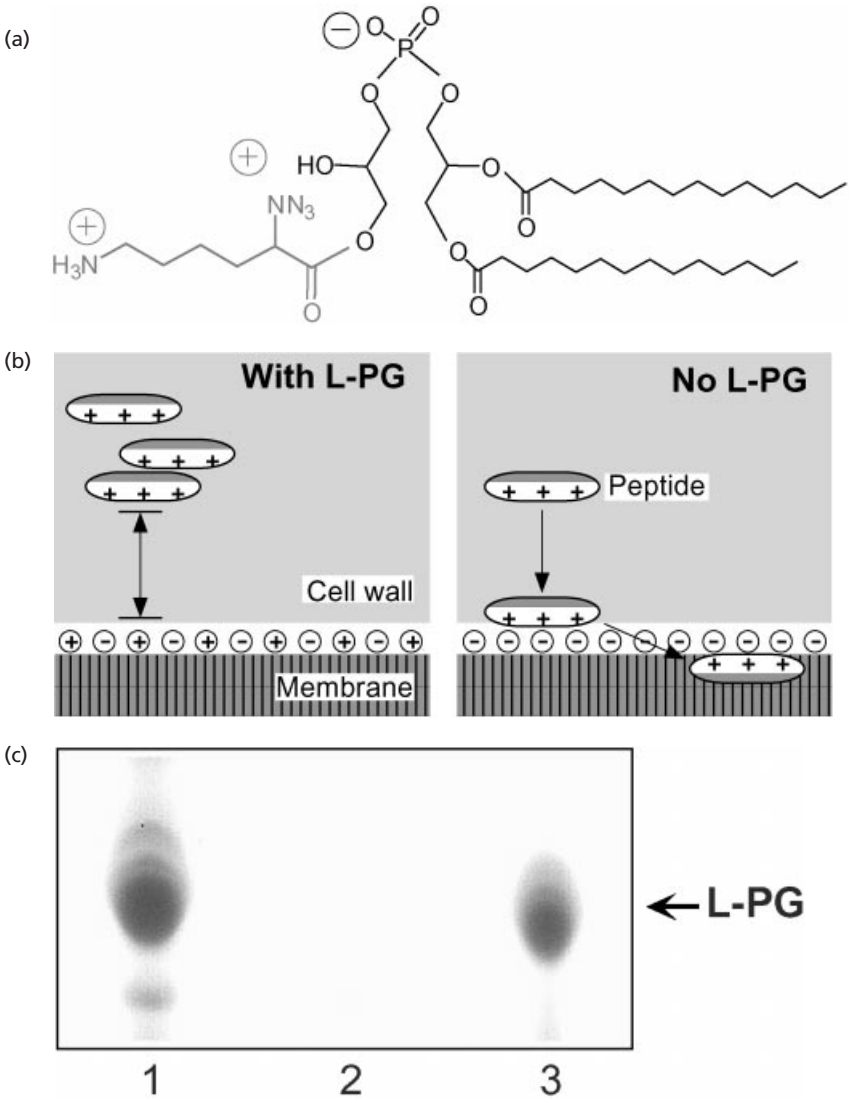

Fig. 1. (a) Structure of lysylphosphatidylglycerol (L-PG). The L-lysine is shown in grey. (b) L-PG-mediated resistance against CAMPs is based on electrostatic repulsion from the bacterial membrane. Modified with permission from (4). (c) Production of L-PG by *Bacillus subtilis* (lane 1) *Staphylococcus aureus* (lane 3) and an *S. aureus mprF* mutant (lane 2). Polar lipids were prepared, separated by TLC and stained with ninhydrin as described recently (5).

alanine (6) and of lipid A with cationic aminoarabinose (2) in Gram-positive and Gram-negative bacteria, respectively, has been reported. Moreover, members of both bacterial groups modify anionic phospholipids of the cytoplasmic membrane with L-lysine, which counteracts the binding and damage of membrane integrity by CAMPs (Fig. 1a, b) (5). The *mprF* gene responsible for synthesis of lysylphosphatidylglycerol (L-PG) in *Staphylococcus aureus* has recently been identified. It encodes a large membrane protein without similarity to proteins of known function (5). *mprF*-related genes occur in the genomes of several human, animal and plant pathogens, where they are assumed to play similar roles in protecting against CAMPs.

Analysis of the *Bacillus subtilis* genome sequence revealed two adjacent and overlapping genes, *yfiW* and *yfiX*, whose products are 52 and 57% similar to the N- and C-terminal portions of MprF, respectively. We hypothesized that the apparent separation into two genes may be a result of sequencing ambiguities and resequenced the region of DNA covering the overlapping section of the two genes in *B. subtilis* type strain ATCC 6051 (Marburg strain). We found one misidentified cytidine nucleotide at position 916 935. Its deletion from the sequence led to the fusion of *yfiW* and *yfiX*, thereby creating a virtually intact *mprF* homologue. Moreover, *B. subtilis* ATCC 6051 produces L-PG (Fig. 1c), which is in accordance with reports from the 1960s on the presence of lysine-containing lipids in *B. subtilis* (1).

Bacillus strains are known to produce a great variety of CAMP-like bacteriocins, such as lantibiotics and lipopeptides (3), probably as a means to limit the growth of other soil organisms. L-PG likely protects *B. subtilis* against its own CAMPs and against those produced by competing microorganisms. Moreover, we identified *mprF*-related genes in the genomes of many other soil microorganisms, such as streptomycetes, pseudomonads, listerias, mycobacteria and even in the archaeon *Methanosarcina barkeri*. In soil inhabitants occasionally causing infections in humans, such as *Bacillus anthracis*, *Clostridium perfringens*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Mycobacterium bovis* the *mprF* homologues may play an additional role in the ability to infect human tissues. These data may stimulate studies on the ecological role of bacteriocins and bacterial countermeasures and lead to a better understanding of the microbial battles taking place in densely colonized habitats.

Petra Staubitz and Andreas Peschel

Microbial Genetics, Tübingen University, D-72076 Tübingen, Germany.
Author for correspondence: Andreas Peschel. Tel: +49 7071 297 7623. Fax: +49 7071 29 5065. e-mail: andreas.peschel@uni-tuebingen.de

1. Bishop, D. G., Rutberg, L. & Samuelsson, B. (1967). The chemical composition of the cytoplasmic membrane of *Bacillus subtilis*. *Eur J Biochem* **2**, 448–453.
2. Gunn, J. S., Lim, K. B., Krueger, J., Kim, K., Guo, L., Hackett, M. & Miller, S. I. (1998). PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. *Mol Microbiol* **27**, 1171–1182.
3. Jack, R. W., Bierbaum, G. & Sahl, H.-G. (1998). *Lantibiotics and Related Peptides*. Berlin: Springer.
4. Peschel, A. (2002). How do bacteria resist human antimicrobial peptides? *Trends Microbiol* **10**, 179–186.
5. Peschel, A., Jack, R. W., Otto, M. & 9 other authors (2001). *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with L-lysine. *J Exp Med* **193**, 1067–1076.
6. Peschel, A., Otto, M., Jack, R. W., Kalbacher, H., Jung, G. & Götz, F. (1999). Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins and other antimicrobial peptides. *J Biol Chem* **274**, 8405–8410.
7. Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature* **415**, 389–395.

Genotypic vs. phenotypic biodiversity in *Lactococcus lactis*

The recent comment article by Panoff *et al*. (4) raises interesting aspects of the taxonomy and ecology of *Lactococcus lactis*, best known for its role as a starter culture in the production of diverse dairy products. The taxonomic structure of *L. lactis* is unusual (5). Genotypically the species is divided into two subspecies (*L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*) based on the sequence of the 16S rRNA genes, which differ by 9–10 bp in the V1 region (7). Historically, three industrially significant phenotypes (lactis, cremoris and diacetylactis) have been recognized. Differentiation of the cremoris and lactis phenotypes is generally based on a few characteristics (production of ammonia from arginine, growth temperature and salt tolerance), while strains of *L. lactis* subsp. *lactis* biovar *diacetylactis* are characterized by their ability to metabolize citrate. Curiously, the genotypic and phenotypic results do not completely correspond, so that strains may have a *lactis* genotype with a cremoris phenotype and *vice versa* (5, 6).

We have used pulsed-field gel electrophoresis of *Sma*I and I-*Ceu*I digests of genomic DNA to examine the genetic diversity of approximately 500 *L. lactis* strains including wild-type lactococci and starter strains used for different purposes in the dairy industry, and have classified these strains as follows:

[1] *L. lactis* subsp. *lactis* (lactis phenotype). The most common group, found in a wide variety of environments and showing considerable metabolic diversity. Some strains are used as dairy starter cultures, and dairy isolates have adapted to growth in milk by acquisition of plasmids or other mobile genetic elements. Similar mobile genetic elements are also found in strains isolated from plant environments (1).

[2] *L. lactis* subsp. *lactis* (diacetylactis phenotype). This group is distinguished because of their ability to metabolize citrate to give the important flavour compound diacetyl. The genetics of this process are complex as most of the citrate-metabolizing genes are chromosomal while the necessary permease is plasmid encoded. They are commonly used as dairy starters but appear to occur rarely in the environment. There is evidence that environmental citrate-utilizing strains lack the citrate permease plasmid and use a different mechanism for citrate uptake (2). We have found that almost all of the industrially used strains are related but show a variety of chromosomal insertions and deletions. The widely studied and sequenced strain IL1403 belongs to this group as its parent strain (CNRZ157) is able to metabolize citrate because of the presence of the citrate permease plasmid.

[3] *L. lactis* subsp. *lactis* (cremoris phenotype). Strains of this type occur rarely and require genotypic analysis to differentiate them from strains of the *cremoris* subspecies.

[4] *L. lactis* subsp. *cremoris* (lactis phenotype). Strains of this type are found commonly but in low numbers in dairy and plant environments, but require genotypic analysis to differentiate them from strains of subsp. *lactis*. This group is of significance as it includes strain MG1363 which has been used for much of the biochemical and genetic research on *L. lactis*.

[5] *L. lactis* subsp. *cremoris* (cremoris phenotype). These are widely used as dairy starters and are recognized as the best cultures for Cheddar cheese manufacture (6). These strains appear totally adapted to the dairy environment and do not survive outside it. Genetic methods for these strains are not well developed, and they are frequently genetically unstable. PFGE analysis of starter cultures separates them into a small number of groups of related strains.

Consequently, we disagree with the statement that *L. lactis* subsp. *cremoris* is growing scarce or has an undefined original habitat (4). It is strains with the cremoris phenotype that are difficult to find in the wild. The reason for this may be that this phenotype has arisen as an adaptation to the milk environment in strains with a long history of use as starter cultures. Knowledge of the genetic basis for the cremoris phenotype, in particular the genes for arginine metabolism, is required to understand why these strains behave as they do. Absence of arginine metabolism could result from the genes for the arginine deiminase pathway being missing or inactivated in these strains. Alternatively, the gene for carbamoyl-phosphate synthetase may be missing or inactive, as is the case in *Lactobacillus delbrueckii* subsp. *lactis* (3) where arginine is required for pyrimidine synthesis, rather than ATP production.

In conclusion, *Lactococcus lactis* consists of two subspecies which occupy similar environments, although genotypic *lactis* strains appear to be more prevalent. Wild type strains of both subspecies show the *lactis* phenotype, while the cremoris and diacetylactis phenotypes are mainly found in dairy starter cultures. The majority of strains used as dairy starters can be grouped into a small number of genetic lineages, and are not representative of the diversity found in *Lactococcus lactis* as a whole.

William Kelly[1] and Lawrence Ward[2]

[1]Horticulture and Food Research Institute, PB 11030, Palmerston North, New Zealand.

[2]Fonterra Research Centre, PB 11029, Palmerston North, New Zealand.

Author for correspondence: William Kelly. Tel: +64 6 356 8080. Fax: +64 6 351 7004. e-mail: wkelly@hortresearch.co.nz

1. Kelly, W. J., Davey, G. P. & Ward, L. J. H. (1998). Conjugative transfer of raffinose metabolism in *Lactococcus lactis*. *FEMS Microbiol Lett* **167**, 145–149.
2. Klijn, N., Weerkamp, A. H. & De Vos, W. E. (1995). Detection and characterization of lactose-utilizing *Lactococcus* spp. in natural ecosystems. *Appl Environ Microbiol* **61**, 788–792.
3. Nicoloff, H., Hubert, J.-C. & Bringel, F. (2001). Carbamoyl-phosphate synthetases (CPS) in lactic acid bacteria and other Gram-positive bacteria. *Lait* **81**, 151–159.
4. Panoff, J.-M., Desmasures, N., Thammavongs, B. & Guéguen, M. (2002). *In situ* protection of microbiodiversity is under consideration. *Microbiology* **148**, 625.
5. Tailliez, P., Tremblay, J., Ehrlich, S. D. & Chopin, A. (1998). Molecular diversity and relationship within *Lactococcus lactis*, as revealed by randomly amplified polymorphic DNA (RAPD). *Syst Appl Microbiol* **21**, 530–538.
6. Urbach, E., Daniels, B., Salama, M. S., Sandine, W. E. & Giovannoni, S. J. (1997). The *ldh* phylogeny for environmental isolates of *Lactococcus lactis* is consistent with rRNA genotypes but not with phenotypes. *Appl Environ Microbiol* **63**, 694–702.
7. Ward, L. J. H., Brown, J. C. S. & Davey, G. P. (1998). Two methods for the genetic differentiation of *Lactococcus lactis* ssp. *lactis* and *cremoris* based on differences in the 16S rRNA gene sequence. *FEMS Microbiol Lett* **166**, 15–20.