Protein Engineering, Design & Selection vol. 21 no. 2 pp. 121–125, 2008
Published online January 19, 2008 doi:10.1093/protein/gzm085

SHORT COMMUNICATION

Engineering *Escherichia coli* heat-resistance by synthetic gene amplification

Daniel Christ[1,2,3] and Jason W. Chin[1,3]

[1]MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK and [2]Present address: Garvan Institute of Medical Research, Victoria Road, Darlinghurst, Sydney NSW 2010, Australia

[3]To whom correspondence should be addressed. E-mail: d.christ@garvan.org.au (D.C.); E-mail: chin@mrc-lmb.cam.ac.uk (J.W.C.)

Organisms have evolved to exploit new environments by processes that involve both mutations and gene amplifications. Though in some cases amplified genes mutate to perform a different molecular function, in other cases altering gene copy number alone is sufficient to change organism function. Here we selected a library of genes, provided at high copy number, for their ability to confer survival on *Escherichia coli* cells at un-physiologically high temperatures. We find that a single gene (*evgA*), encoding a master transcriptional regulator, is overwhelmingly selected and allows survival upon heating to temperatures in excess of 50°C. While the detailed mechanisms of this resistance remained unclear, our results demonstrate the potential of copy number manipulation for the engineering of organisms

Keywords: copy number variation/synthetic biology/heat-resistance

Introduction

Gene amplification is the process by which a DNA segment is iterated to provide multiple copies of the DNA segment per cell. Though gene amplification was first observed in *Escherichia coli* over 40 years ago (Horiuchi *et al*., 1962, 1963), the full extent of gene amplification, and the resulting copy number variation, in determining phenotypes across organisms is only just beginning to be appreciated. Gene amplification can contribute to tumorigenesis (Pollack *et al*., 1999), antibiotic resistance in microbes (Nilsson *et al*., 2006) and is causal to a range of neurological conditions (Niebuhr, 1978; Ledbetter *et al*., 1981; Hassold and Jacobs, 1984). Gene amplification can underlie adaptive evolution (Hastings *et al*., 2004; Kugelberg *et al*., 2006; Roth *et al*., 2006; Slack *et al*., 2006) and contribute to new gene function and genome evolution (Luscombe *et al*., 2004; Teichmann and Babu, 2004). Moreover, gene amplification contributes to the considerable gene copy number variation within the human population (Iafrate *et al*., 2004, Sebat *et al*., 2004) that may affect the variability of our phenotypic responses to a range of diseases, therapeutic agents and environmental cues (Gonzalez *et al*., 2005). Our emerging understanding of the role of copy number variation in determining organism function suggests that synthetic alteration of gene copy number, coupled with genetic selection may provide access to organisms with improved survival under extreme conditions.

Although gene copy number effects have been studied ever since plasmid-based genomic libraries became available in the mid 1970s (Clarke and Carbon, 1975), most studies have focussed on either resistance to antibiotic and cytotoxic agents (Chen and Bishai, 1998; Cotrim *et al*., 1999; Burger *et al*., 2000) or on limited variations of physiological conditions. For instance, a large body of previous work has explored cellular mechanisms for dealing with heat shock at elevated but non-lethal temperatures (Yura *et al*., 1993). In response to increase the temperature of *E.coli* growth from 37 to 42°C (a stressful but permissive temperature), σ_H and σ_E dependent transcription is activated (Yura *et al*., 1993; Lund, 2001). The molecular mechanisms by which many of the proteins encoded by heat inducible transcripts, including chaperones and operate have been extensively investigated (Arsene *et al*., 2000). In contrast, there has been a little work exploring the factors that might allow cells to survive at even higher temperatures, well beyond their normal physiological limits. Yeh *et al*. (1997) over-expressed a heterologous heat shock protein in *E.coli* and reported a modest effect on the thermal resistance (10^1- to 10^2-fold improvement in survival after 1 h at 47.5°C). Recently, Park *et al*. (2005) focussed on creating thermally resistant *E.coli* using a library of engineered zinc fingers aimed at repressing gene expression. A library of cells expressing zinc fingers, with a range of DNA binding specificities, was incubated for 2 h at 50°C, and zinc fingers able to confer a 10^2-fold increase in thermal resistance, after 2 h at 50°C, isolated. Lenski and co-workers have investigated the ability of *E.coli* strains propagated for many generations at 41.2°C to survive at 50°C, after pre-incubation at 41.2°C—which may allow for epigenetic change—they find that cells can survive up to 10^2-fold better after incubation for 4 h at 50°C (Riehle *et al*., 2003).

Here, we ask whether amplification of any gene, from a library of *E.coli* genes provided extra chromosomally at high copy number, can facilitate survival at high and otherwise lethal temperatures. We find that a single gene (*evgA*), encoding a master transcriptional regulator is overwhelmingly selected and allows the mesophilic gut bacterium *E.coli* to survive heating to temperatures in excess of 50°C. Our results demonstrate the potential of copy number manipulation for the engineering of organisms.

Methods

Construction of an E.coli gene library

We fragmented genomic DNA of *E.coli* W3110 in a Nebulizer DNA fragmentation device (Invitrogen), predominantly to about 300–1200 bp. We ligated oligonucleotide adapters to the fragments and ligated the resulting inserts into plasmid pW656 (as previously described, Christ and Winter (2006)). Plasmid pW656 is derived from pHEN1

© The Author 2008. Published by Oxford University Press. All rights reserved.
For Permissions, please e-mail: journals.permissions@oxfordjournals.org

(Hoogenboom *et al*., 1991) and carries an ampicillin resistance marker as well as the pUC origin of replication (300–500 copies per cell). After transformation into *E.coli* TG1 (Gibson, 1984), we obtained 5×10^6 transformants, from which we obtained plasmid DNA using a DNA miniprep kit (Qiagen). For selection, we transformed the plasmid DNA into *E.coli* TG1 yielding 1×10^8 independent transformants (and >99% library coverage by Poisson distribution).

Selection of amplified E.coli genes that confer thermal resistance

We grew cells in 2xTY broth supplemented with 100 μg/ml ampicillin at 37°C for 3 h starting from an initial OD600 of 0.1. We diluted cells 10-fold into 2xTY broth and heated them in an Eppendorf Mastercycler gradient PCR machine in a 96-well plate for 2 h (in a 100 μl volume). After the heat selection step, we plated cells on 2xTY agar plates supplemented with 100 μg/ml ampicillin and 2% glucose. We visually inspected the plates the next day after incubation at 37°C overnight. Selection temperatures in the first round were as follows: 40.7°C, 41.7°C, 43.5°C, 45.9°C, 48.7°C, 51.6°C, 54.4°C, 56.9°C, 58.9°C, 60.1°C and 60.3°C. Selection temperatures in second round were 47.5°C, 48.0°C, 48.6°C, 49.4°C, 50.3°C, 51.1°C, 51.9°C, 52.5°C and 52.9°C.

Analyses of selection

We extracted plasmid DNA after each round of selection using a DNA miniprep kit (Qiagen) and amplified the inserts by PCR using primers 5′-GCT TAT CAG ACC TTT ACA-3′ and 5′-CCC TCA TAG TTA GCG TAA CGA-3′. We resolved the resulting products by agarose gel electrophoresis (PCR products exceed plasmid insert sizes by approximately 300 bp due to the amplification of flanking sequences). To analyze the heat resistance of individual clones, we transformed plasmid DNA into *E.coli* TG1 and plated on 2xTY agar plates supplemented with 100 μg/ml ampicillin and 2% glucose. We grew cultures from individual colonies in 2xTY broth supplemented with 100 μg/ml ampicillin and 4% glucose at 37°C. The next day, cells we the diluted cells and heated them in a gradient PCR machine (as described above).

Construction of D52 N mutant

We amplified fragments of the *evgA* gene by PCR from plasmid preparations of clone evgAf using primer pairs (I) 5′-GCT TAT CAG ACC TTT ACA-3′, 5′-TGA CGA TAT CAG GCT TAA GTG TTT CCA C-3′ and (II) 5′-ATG AGA TAT CGT CAT CAT TAA TGT CGA CAT CCC CGG AG-3′, 5′-CCC TCA TAG TTA GCG TAA CGA-3′. We digested the amplified fragments with *Eco*RV and either *Xho*I (fragment I) or *Rsr*II (fragment II) restriction endonuclease, ligated into *Xho*I/*Rsr*II digested plasmid pW656 and transformed the resulting construct into *E.coli* TG1.

Results

Selection of amplified E.coli *genes conferring thermal resistance*

To select for genes that, when provided at amplified copy number, confer thermal resistance (Fig. 1 schematic) we first transformed a library of high-copy-plasmid borne *E.coli* genomic fragments into *E.coli* cells. After growth in liquid media, the cells were heated for 2 h at temperatures ranging from 40°C to 60°C at 1–2.8°C intervals. Approximately 10^3 cells were obtained after heating at 48.7°C, while no colonies were obtained at higher temperatures in this first round of selection.

Plasmid DNA was isolated from cells surviving at 48.7°C and used to retransform fresh *E.coli* cells. The retransformed cells were used to inoculate liquid media and a second round of thermal selection carried out with heating at 47–53°C for 2 h (0.4–0.9°C steps were used). After the second round of selection, we obtained 31 colonies after heating at 50.3°C, whereas no colonies were obtained for the vector control. No colonies were obtained when heating the library at higher temperature while the next lower selection temperature (49.4°C) yielded ~10^3 colonies. All 31 colonies from the

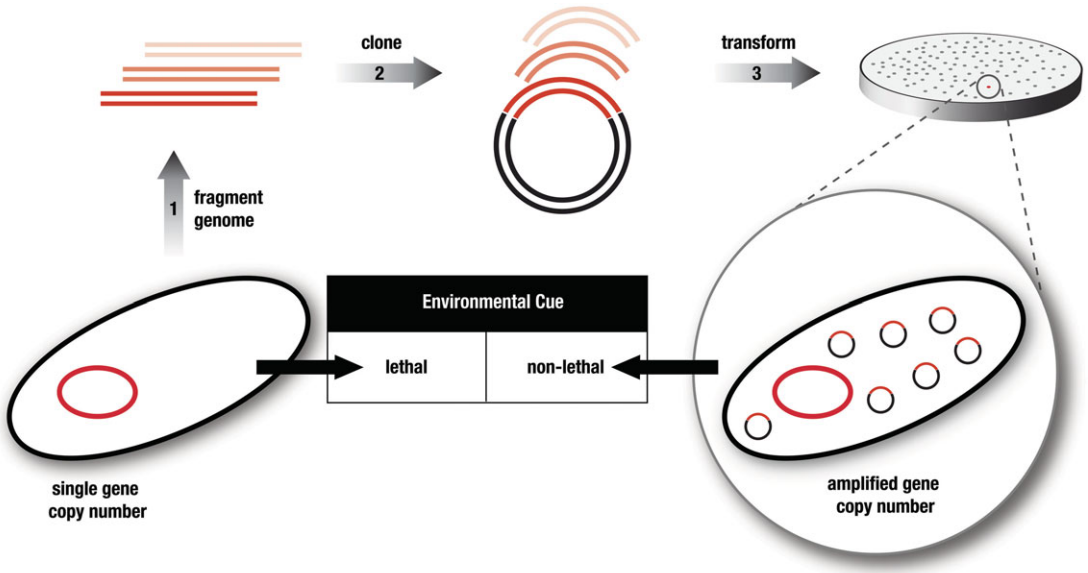

Fig. 1. Selection for heat-resistance. Genomic DNA is fragmented (1) and cloned into a high-copy number plasmid (2). After transformation (3), cells harbouring amplified genes are selected by heating at high and otherwise lethal temperature.

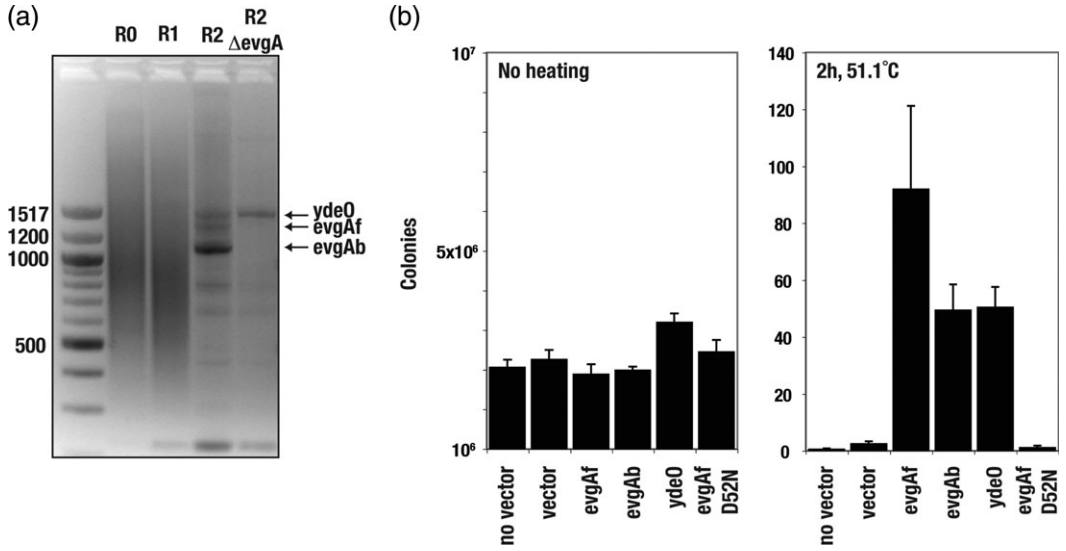

Fig. 2. (**A**) Insert size distribution of library before (R0) and after one (R1, 48.7°C) and two (R2, 49.4°C) rounds of heat selection (inserts were amplified by PCR). Rightmost lane (R2, ΔevgA) shows distribution after two rounds of selection and removal of the dominant clone. (**B**) Clones evgAf, evgAb, ydeO, but not background vector or evgAfD52 N display increased cell survival after 2 h of heating at 51.1°C ($n = 6$).

50.3°C selection step were sequenced of which 23 yielded analyzable DNA sequences. This revealed that only two clones harbouring a single gene, encoding the transcriptional activator EvgA (Utsumi *et al*., 1992), had been selected. Although both clones encode the complete EvgA open-reading frame, they have the opposite orientation in respect to the library plasmid (clone evgAf: W3110 2488937–2490052, 11 × observed; clone evgAb: W3110 2489885–2489013, 12 × observed). This strongly suggested that the two rounds of selection were sufficient to provide convergence in the library.

To examine the course of the selection for thermal resistance, we prepared DNA from the plasmid populations before selection and after each round of selection (Fig. 2A) and examined the distribution of clone size by PCR. Before any selection on the pool the plasmid inserts form a broad smear with no single clone dominant. After one round of selection (at 48.7°C), the distribution is similar to that before selection. However, after the second round of selection (at 49.4°C), two bands of ∼1300 and 1100 bp dominate. These bands correspond to the insert sizes of the EvgA encoding clones evgAf and evgAb. In order to analyze whether any additional clones had been selected we digested the template DNA with a restriction endonuclease (*Eco*RV) cleaving a 6 bp recognition site within the *evgA* gene. After PCR amplification of the digested preparation (Fig. 2A; R2 ΔevgA) a single band of about 1500 bp was observed (Fig. 2A; ydeO). We sequenced 16 clones after transformation of the preparation into *E.coli* TG1 of which (8/16) had an identical insert (W3110 1585694–1584416), encoding the transcriptional activator YdeO, which is known to be directly regulated by EvgA (Masuda and Church, 2003).

Synthetic evgA amplification confers thermal resistance

To confirm the effect of the selected clones on heat resistance, plasmid preparations were prepared and transformed into *E.coli* and survival was examined after heating. Considering the highest temperature under which we had observed colonies in the selection (50.3°C), we tested the next temperature step (51.1°C) to see if we would observe survival under this condition. This experiment confirmed that amplification of both *evgA* and *ydeO* significantly increased the thermal resistance (Fig. 2B). We also analyzed whether, in the case of the *evgA* gene, the observed effect was directly linked to the encoded transcription factor (rather than an effect mediated by DNA or RNA elements). For this

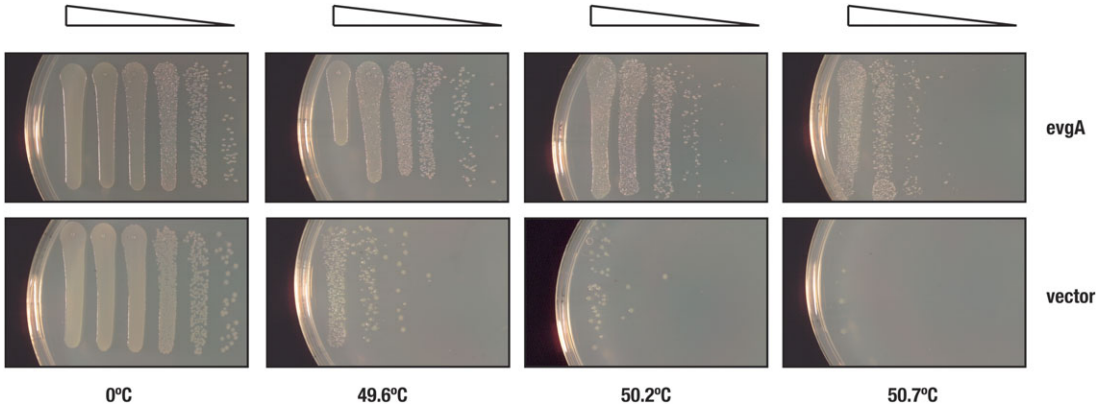

Fig. 3. Amplification of *evgA* gene increases cell survival over a range of elevated temperatures (upper panels) compared to vector control (lower panels). Dilution steps within each panel are 10-fold. Cells were heated for 2 h.

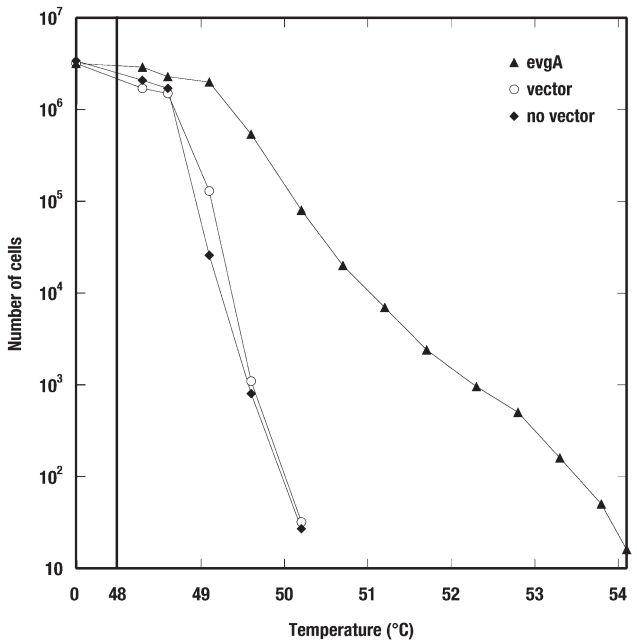

Fig. 4. Temperature dependence of cell survival. Shown are amplified *evgA* (triangle), background vector (circle) and untransformed cells (diamond). Cells were heated for 2 h.

purpose, we introduced a conservative aspartate to asparagine change into EvgA at position 52, a mutation, which had previously been shown to abolish EvgA activity *in vivo* (Utsumi *et al*., 1994). The experiment revealed that the D52 N mutation indeed completely abolished the heat resistant phenotype (Fig. 2B).

To further investigate the extent to which *evgA* amplification confers thermal resistance on *E.coli*, survival at a wider temperature range (48.3–54.1°C) was examined. For this purpose, clone evgAf and the background pW656 vector were transformed into *E.coli* and compared to wild-type bacteria. Outcomes of a typical heating experiment are shown in Figs 3 and 4. Cells transformed with the background vector or untransformed cells show a steep and comparable temperature dependent death (Fig. 4) indicating that neither plasmid propagation nor the presence of ampicillin for plasmid maintenance had a measurable effect on thermal resistance. In contrast, cells transformed with plasmid encoded EvgA show enhanced thermal resistance over a range of temperatures and the temperature dependent thermal resistance curve has a shallower slope that of wild-type cells (Fig. 4).

Discussion

EvgA is master transcriptional regulator, and part of a two-component signal transduction system in enterobacteria (Stock *et al*., 1989; Perraud *et al*., 1998). It is activated by the sensor protein and histidine kinase EvgS on Asp 52 (Perraud *et al*., 1998) in response low pH, a physiological condition in enterobacteria encountered during their passage through the stomach. Previous work by Masuda and Church (2002, 2003) has shown that EvgA over-expression upregulates the transcription of 37 genes. Fifteen of these genes are upregulated directly by EvgA, whereas the remaining 22 are regulated indirectly through YdeO. EvgA, YdeO, and genes they regulate were shown to increase survival under acidic conditions (Masuda and Church, 2002, 2003). Since we find that, at elevated temperatures, *evgA* amplification is strongly selected and *ydeO* amplification is the next most strongly selected we suggest that the transcriptional network that confers thermal resistance overlaps with, and may be identical to, that turned on in response to acidic conditions. Since a conservative mutation (D52 N) of the phosphorylation site on plasmid encoded EvgA destroys thermal resistance, this would suggest that, in the case of *evgA* amplification, phosphorylation is still required for activation of downstream genes. Overlapping acid and thermal resistance pathway regulation may provide part of a mechanistic explanation for the observation that prior exposure of *E.coli* to acid stress confers increased subsequent thermal resistance (this has long been know by food engineers and complicates the pasteurization of grape juice) (Cheng *et al*., 2002).

The genes activated by EvgA include a number involved in periplasmic function—including periplasmic chaperones—as well as in membrane and permeability functions (Masuda and Church, 2002, 2003). This is in agreement with Lenski and co-workers experiments with long-term exposure to elevated temperatures that demonstrate the importance of extra-cytoplasmic stress response for prolonged incubation at elevated and lethal temperatures (Riehle *et al*., 2003).

Heating places thermal stress on every molecule in the cell, and therefore requires a response that is able to compensate for multiple potentially catastrophic defects in molecular function. Our results suggest that a complex molecular problem such as this can be effectively addressed by amplification of a single master transcriptional regulator. Thus, our experiments indicate that *evgA* amplification can confer significant thermal resistance on *E.coli* cells (10^2- to 10^4-fold increase in survival observed).

It will be interesting to examine the range of phenotypes that can be accessed by gene amplification, to investigate whether natural gene amplification hotspots for complex phenotypes correlate with the amplification of master regulators, and to quantitatively investigate how gene amplification alters the dynamics of gene regulation (Shen-Orr *et al*., 2002; Mangan *et al*., 2003) and the cell-to-cell variability in protein copy number (Rosenfeld *et al*., 2005).

Acknowledgements

D.C. was a Fellow of Trinity College (Cambridge University) throughout the course of this work. J.W.C. is an EMBO Young Investigator.

Funding

This work was funded by the Medical Research Council.

References

Arsene,F., Tomoyasu,T. and Bukau,B. (2000) *Int J Food Microbiol.*, **55**, 3–9.

Burger,H., Capello,A., Schenk,P.W., Stoter,G., Brouwer,J. and Nooter,K. (2000) *Biochem. Biophys. Res. Commun.*, **269**, 767–774.

Chen,P. and Bishai,W.R. (1998) *Infect. Immun.*, **66**, 5099–5106.

Cheng,H.Y., Yang,H.Y. and Chou,C.C. (2002) *J. Food Prot.*, **65**, 260–265.

Christ,D. and Winter,G. (2006) *J. Mol. Biol.*, **358**, 364–371.

Clarke,L. and Carbon,J. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 4361–4365.

Cotrim,P.C., Garrity,L.K. and Beverley,S.M. (1999) *J. Biol. Chem.*, **274**, 37723–37730.

Gibson,T.J. (1984) *Studies on the Epstein-Barr virus genome.* PhD thesis, University of Cambridge, Cambridge.
Gonzalez,E., *et al*. (2005) *Science*, **307**, 1434–1440.
Hassold,T.J. and Jacobs,P.A. (1984) *Annu. Rev. Genet.*, **18**, 69–97.
Hastings,P.J., Slack,A., Petrosino,J.F. and Rosenberg,S.M. (2004) *PLoS Biol.*, **2**, e399.
Hoogenboom,H.R., Griffiths,A.D., Johnson,K.S., Chiswell,D.J., Hudson,P. and Winter,G. (1991) *Nucleic Acids Res.*, **19**, 4133–4137.
Horiuchi,T., Horiuchi,S. and Novick,A. (1963) *Genetics*, **48**, 157–169.
Horiuchi,T., Tomizawa,J.I. and Novick,A. (1962) *Biochim. Biophys. Acta*, **55**, 152–163.
Iafrate,A.J., Feuk,L., Rivera,M.N., Listewnik,M.L., Donahoe,P.K., Qi,Y., Scherer,S.W. and Lee,C. (2004) *Nat. Genet.*, **36**, 949–951.
Kugelberg,E., Kofoid,E., Reams,A.B., Andersson,D.I. and Roth,J.R. (2006) *Proc. Natl. Acad. Sci. USA*, **103**, 17319–17324.
Ledbetter,D.H., Riccardi,V.M., Airhart,S.D., Strobel,R.J., Keenan,B.S. and Crawford,J.D. (1981) *N. Engl. J. Med.*, **304**, 325–329.
Lund,P.A. (2001) *Adv. Microb. Physiol.*, **44**, 93–140.
Luscombe,N.M., Babu,M.M., Yu,H., Snyder,M., Teichmann,S.A. and Gerstein,M. (2004) *Nature*, **431**, 308–312.
Mangan,S., Zaslaver,A. and Alon,U. (2003) *J. Mol. Biol.*, **334**, 197–204.
Masuda,N. and Church,G.M. (2002) *J. Bacteriol.*, **184**, 6225–6234.
Masuda,N. and Church,G.M. (2003) *Mol. Microbiol.*, **48**, 699–712.
Niebuhr,E. (1978) *Hum. Genet.*, **44**, 227–275.
Nilsson,A.I., Zorzet,A., Kanth,A., Dahlstrom,S., Berg,O.G. and Andersson,D.I. (2006) *Proc. Natl. Acad. Sci. USA*, **103**, 6976–6981.
Park,K.S., Jang,Y.S., Lee,H. and Kim,J.S. (2005) *J. Bacteriol.*, **187**, 5496–5499.
Perraud,A.L., Kimmel,B., Weiss,V. and Gross,R. (1998) *Mol. Microbiol.*, **27**, 875–887.
Pollack,J.R., Perou,C.M., Alizadeh,A.A., Eisen,M.B., Pergamenschikov,A., Williams,C.F., Jeffrey,S.S., Botstein,D. and Brown,P.O. (1999) *Nat. Genet.*, **23**, 41–46.
Riehle,M.M., Bennett,A.F., Lenski,R.E. and Long,A.D. (2003) *Physiol. Genomics*, **14**, 47–58.
Rosenfeld,N., Young,J.W., Alon,U., Swain,P.S. and Elowitz,M.B. (2005) *Science*, **307**, 1962–1965.
Roth,J.R., Kugelberg,E., Reams,A.B., Kofoid,E. and Andersson,D.I. (2006) *Annu. Rev. Microbiol.*, **60**, 477–501.
Sebat,J., *et al*. (2004) *Science*, **305**, 525–528.
Shen-Orr,S.S., Milo,R., Mangan,S. and Alon,U. (2002) *Nat. Genet.*, **31**, 64–68.
Slack,A., Thornton,P.C., Magner,D.B., Rosenberg,S.M. and Hastings,P.J. (2006) *PLoS Genet.*, **2**, e48.
Stock,J.B., Ninfa,A.J. and Stock,A.M. (1989) *Microbiol. Rev.*, **53**, 450–490.
Teichmann,S.A. and Babu,M.M. (2004) *Nat. Genet.*, **36**, 492–496.
Utsumi,R., Katayama,S., Ikeda,M., Igaki,S., Nakagawa,H., Miwa,A., Taniguchi,M. and Noda,M. (1992) *Nucleic Acids Symp. Ser.*, 149–150.
Utsumi,R., Katayama,S., Taniguchi,M., Horie,T., Ikeda,M., Igaki,S., Nakagawa,H., Miwa,A., Tanabe,H. and Noda,M. (1994) *Gene*, **140**, 73–77.
Yeh,C.H., Chang,P.F., Yeh,K.W., Lin,W.C., Chen,Y.M. and Lin,C.Y. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 10967–10972.
Yura,T., Nagai,H. and Mori,H. (1993) *Annu. Rev. Microbiol.*, **47**, 321–350.

Received August 28, 2007; revised October 22, 2007; accepted November 22, 2007

Edited by Leo James