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# Cationic antimicrobial peptides elicit a complex stress response in *Bacillus subtilis* that involves ECF-type sigma factors and two-component signal transduction systems

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Stress responses of *Bacillus subtilis* to membrane-active cationic antimicrobial peptides were studied. Global analysis of gene expression by DNA macroarray showed that peptides at a subinhibitory concentration activated numerous genes. A prominent pattern was the activation of two extracytoplasmic function sigma factor regulons, SigW and SigM. Two natural antimicrobial peptides, LL-37 and PG-1, were weak activators of SigW regulon genes, whereas their synthetic analogue poly-L-lysine was clearly a stronger activator of SigW. It was demonstrated for the first time that LL-37 is a strong and specific activator of the YxdJK two-component systems, one of the three highly homologous two-component systems sensing antimicrobial compounds. YxdJK regulates the expression of the YxdLM ABC transporter. The LiaRS (YvqCE) TCS was also strongly activated by LL-37, but its activation is not LL-37 specific, as was demonstrated by its activation with PG-1 and Triton X-100. Other strongly LL-37-induced genes included *yrhH* and *yhcGHI*. Taken together, the responses to cationic antimicrobial peptides revealed highly complex regulatory patterns and induction of several signal transduction pathways. The results suggest significant overlap between different stress regulons and interdependence of signal transduction pathways mediating stress responses.

## INTRODUCTION

Our purpose in this study was to characterize stress responses of *Bacillus subtilis* to cationic antimicrobial peptides. Antimicrobial peptides represent an ancient form of weapon in host defence mechanisms and their ubiquitous existence in cells and organisms of all types suggests important roles for them in innate immunity and defence against microbial invasion (reviewed by, for example, Yeaman & Yount, 2003). Natural antimicrobial peptides are typically amphipathic and positively charged, and they contain well-defined $\alpha$-helical or $\beta$-sheet structures (see also below). Mammalian antimicrobial peptides include defensins, protegrins and cathelicidins. Antimicrobial peptides typically attach to membrane surfaces of invading pathogens and disturb membrane integrity (Zasloff, 2002). In the case of nisin and epidermidin, however, a specific target has been identified. They interact with the membrane-bound peptidoglycan precursors and disturb cell wall biosynthesis (Breukink *et al.*, 1999; Brotz *et al.*, 1998). Modification of the net negative charge of the bacterial cell surface by adding positively charged residues to teichoic and lipoteichoic acids helps bacteria to avoid being killed by antimicrobial peptides. It has been shown that inactivation of the *dlt* operon of *Staphylococcus aureus* and consequent lack of D-alanine substitution in teichoic and lipoteichoic acids results in increased negative charge of the cell surface and increased sensitivity to defensins, protegrins and other antimicrobial peptides (Peschel *et al.*, 1999). The absence of D-alanine substitution in the anionic polymers of *Listeria monocytogenes* also increases sensitivity to antimicrobial peptides and decreases virulence (Abachin *et al.*, 2002).

Extracytoplasmic function (ECF) sigma factors are regulatory components by which bacteria control gene expression in response to environmental stress. There are seven different ECF-type sigma factors in the Gram-positive model bacterium *B. subtilis* (Helmann, 2002; Kunst *et al.*, 1997). Several stress conditions activate the SigW regulon. These include alkaline shock (Wiegert *et al.*, 2001), inhibition of the cell wall synthesis by antibiotics such as vancomycin and disturbance of the integrity of the cell membrane by detergents (Cao *et al.*, 2002b). The SigW sigma factor is associated with the membrane-bound SigW anti-sigma factor when the *Bacillus* cell is not exposed to environmental

Abbreviations: ECF, extracytoplasmic function; PLL, poly-L-lysine; TCS, two-component system.

stress (Schobel *et al.*, 2004). Under stress conditions, the anti-sigma factor is proteolytically degraded, resulting in the release of SigW from the membrane and binding to gene promoters of the regulon (Schobel *et al.*, 2004). A similar pattern is anticipated for other ECF-type sigma factors. SigM is required for combating stress due to antibiotic effects on the cell wall, ethanol, heat, acid and superoxide (Thackray & Moir, 2003). It is also essential for survival in environments containing high concentrations of salt (Horsburgh & Moir, 1999), suggesting that it is required for maintaining the integrity of the cell envelope. Alternative sigma factors are not the only regulatory systems that are involved in stress tolerance: two-component systems (TCSs) also have a role in controlling gene expression in environmental changes. TCSs are signalling devices composed of a membrane-bound sensor kinase and a response regulator. *B. subtilis* two-component regulation has recently been reviewed (Ogura & Tanaka, 2002).

We studied stress responses to two naturally occurring antimicrobial peptides, LL-37 and PG-1, and their synthetic analogue poly-L-lysine (PLL), using a DNA macroarray and real-time RT-PCR. Human LL-37 is 37 amino acid residues long and belongs to the cathelicidin family of antimicrobial peptides (Johansson *et al.*, 1998; Turner *et al.*, 1998). It is an amphipathic and $\alpha$-helical peptide that probably disrupts the lipid bilayer by a toroidal pore mechanism (Henzler Wildman *et al.*, 2003; Johansson *et al.*, 1998). The porcine protegrin PG-1 is composed of 18 amino acid residues, including four cysteines, and forms a two-stranded antiparallel $\beta$-sheet linked by a $\beta$-turn (Aumelas *et al.*, 1996; Fahrner *et al.*, 1996). The four cysteines of PG-1 form two disulphide bonds, which are important for the $\beta$-sheet conformation and antimicrobial activity (Harwig *et al.*, 1996). The synthetic peptide PLL differs from the natural peptides in that it is not amphipathic. Its mode of action on membranes is unclear.

It was found that the antimicrobial peptides induced ECF-type sigma factor regulons in a complex manner. Several genes that are regulated by two-component signal transduction systems were also induced. Most interestingly, LL-37 strongly upregulated, via the YxdJK TCS, the *yxdLM* genes encoding an ABC-type transporter of unknown function. The *yvcRS* and *bceAB* (*ytsCD*) genes, which encode ABC transporters highly homologous with YxdLM, were also moderately upregulated. Interestingly PG-1, PLL and Triton X-100 did not induce the expression of any of these ABC-transporter genes.

## METHODS

**Bacterial growth conditions.** *B. subtilis* cells were grown in Luria–Bertani (LB) medium or in BFA minimal medium at 37 °C with vigorous shaking. The BFA medium is a modified Spizizen's minimal salts medium (Anagnostopoulos & Spizizen, 1961) containing glutamine instead of ammonium sulphate as the nitrogen source. When needed, kanamycin and erythromycin were added at concentrations of 10 μg ml$^{-1}$ and 1 μg ml$^{-1}$, respectively. *B. subtilis* strain 168 and its derivatives were used in all experiments. The sensitivity of strains to four antimicrobial peptides, HNP-1 (American Peptide Company), PG-1 (Bachem), LL-37 (Ale Närvänen, University of Kuopio, Finland) and PLL (Sigma-Aldrich) were tested with the Bioscreen C Microbiology Reader (Labsystems). The culture volume was 150 μl and the number of bacteria in the inoculum was approximately $10^6$ ml$^{-1}$. These tests were also repeated in shake-flask cultures at approximately $10^8$ bacteria (ml inoculum)$^{-1}$.

**Mutant constructions.** The *sigW*::*neo* and *sigM*::pMUTIN4 mutations were introduced into strains by transformation with chromosomal DNA of the *B. subtilis* strains HB4247 (kindly supplied by J. D. Helmann) and MJH003 (Horsburgh & Moir, 1999), respectively. The *sigW sigM* double mutant was constructed by transforming the strain IH8342 containing the *sigW*::*neo* mutation with the chromosome of MJH003. The *yvcQ* gene was inactivated with pMUTIN4, as described by Vagner *et al.* (1998). The *yxdJ* and *yxdK* null mutations were obtained from Naotake Ogasawara (Nara Institute of Science and Technology, Nara, Japan).

**RNA isolation, labelling with $^{33}$P and DNA macroarray analysis.** For RNA isolations, strains were grown in the BFA minimal medium containing 100 mM NaCl in shake-flask cultures. Cell densities were measured with a Klett colorimeter. Antimicrobial peptides were added at 60 Klett units and samples for RNA isolation were harvested after 20 min from 4 ml cell culture by centrifugation. Control samples without peptides were treated in a similar manner. Cells were resuspended in 400 μl ice-cold culture medium and transferred to screw-capped Eppendorf tubes containing 1·5 g glass beads, 50 μl 10 % SDS, 50 μl 3 M sodium acetate and 500 μl phenol/chloroform/isoamylalcohol (25 : 24 : 1 by vol.). The tubes were frozen in liquid nitrogen, followed by vigorous shaking for 6 min with a face-grinding machine and centrifugation at 10 000 r.p.m. for 5 min. The water phase was mixed (Vortex) with 1 vol. chloroform and centrifuged at 14 000 r.p.m. for 2 min. Next, the water phase was mixed with 2 vols Roche lysis/binding buffer, and the RNA extraction was continued with the Roche High Pure RNA Isolation Kit according to the manufacturer's instructions.

DNA macroarray analysis was carried out using Panorama *B. subtilis* gene array filters and specific cDNA labelling primers (Sigma Genosys). The Panorama *B. subtilis* gene array contains duplicate spots of PCR products representing currently known *B. subtilis* genes. Prior to cDNA synthesis, the quality of RNA was confirmed using Northern blotting. For cDNA synthesis, 10 μg RNA was used, and the synthesis was performed as described by Wiegert *et al.* (2001). The SuperScript II reverse transcriptase was purchased from Gibco-BRL. cDNA was purified with MicroSpin G-25 columns (Amersham Pharmacia Biotech) and the labelling efficiency was determined with a liquid scintillation counter. Prehybridization, hybridization and washing of the filters were performed according to the manufacturer's instructions. The DNA array filters were exposed overnight on phosphor screens and the screens were scanned with a Fluorescent Image Analyser FLA-2000 (Fujifilm). Hybridization signal intensities were quantified with the ArrayVision software (Imaging Research), as described by Wiegert *et al.* (2001). Data were filtered to avoid false positives by excluding genes with a signal-to-noise ratio $<3$ (Array Vision software) and normalized by dividing the intensity of each spot by the mean intensity of all the spots. Each experiment was carried out twice with RNA isolated from two independent cultures. Genes were regarded as induced when the induction ratio was $>2$ in both experiments.

**Quantitative real-time RT-PCR.** For real-time RT-PCR, RNA was isolated similarly as for the DNA array. RT reactions were carried out with the Omniscript Reverse Transcriptase Kit (Qiagen) according to the manufacturer's instructions with the exception of an additional DNase I (Roche) treatment. An equal amount of RNA (2 μg)

was used in each RT reaction. Primers used in RT reactions were random hexamers (0·15 μg $ml^{-1}$) provided by Roche. The absence of chromosomal DNA in the RNA preparations was verified with a control sample that was not treated with RT, but was otherwise treated in a similar manner to the RT-treated samples. Real-time PCR reactions were carried out with specific primer pairs using the SYBR green PCR master mix (Applied Biosystems). Primers were designed with the Primer Express software (Applied Biosystems) and purchased from Sigma Genosys or TAGC Copenhagen. Sequences of the PCR primers for the genes studied are shown in Table 1. The amplification and detection of PCR products were performed with the ABI PRISM 5700 sequence detection system (Applied Biosystems). The cycling conditions were: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The threshold cycle (Ct) is the first cycle at which the fluorescence becomes detectable above the background and is inversely proportional to the logarithm of the initial number of template molecules. Ct values of known quantities of *B. subtilis* chromosomal DNA were plotted for each primer pair to obtain standard curves. The standard curves allowed us to convert the Ct values of each amplified gene in the cDNA preparations to relative numbers of cDNA molecules. These cDNA values were normalized with the value of *gyrA*, which was constant in different growth conditions and phases (data not shown).

## RESULTS

### Optimization of experimental conditions for the transcriptome analysis

Four different types of peptides were chosen to study the response to antimicrobial peptides. Human cathelicidin LL-37 and porcine protegrin PG-1 are cathelin-associated α-helical and β-sheet structures, respectively (Turner *et al.*, 1998), and human defensin HNP-1 belongs to α-defensins with a triple-stranded β-sheet structure (Lehrer & Ganz, 2002). PLL was chosen as a synthetic analogue of cationic peptides (Vaara & Vaara, 1983). These antimicrobial peptides were first used at various concentrations in a Bioscreen assay to determine the range of concentrations needed to inhibit the growth of *B. subtilis*. Then, the effects of peptide concentrations that inhibited growth but did not kill bacteria were determined in shake-flask cultures (Fig. 1). Bacteria were grown in BFA minimal medium containing an additional 100 mM NaCl to enhance the microbicidal effect of the peptides (Turner *et al.*, 1998). No effect on growth was detected with HNP-1 for any of the tested concentrations (up to 9 μg $ml^{-1}$) and it was omitted from further analyses. For RNA isolations, bacteria were cultured in shake flasks and the antimicrobial peptides were added at 60 Klett units at concentrations of 1·5 μM (LL-37), 50 nM (PG-1) or 1 mM (PLL).

### Cationic antimicrobial peptides induce a subset of SigW- and SigM-regulated genes

We studied the effects of the antimicrobial peptides on gene expression in *B. subtilis* using DNA macroarrays containing all the ORFs of the *B. subtilis* genome. Bacteria were exposed to peptide stress for 20 min, after which total RNA was isolated and the DNA macroarray analysis was carried out. In parallel, control cultures without a peptide addition were similarly treated. Two independent array experiments from separate cultures with each peptide treatment were performed. The array data were analysed with the ArrayVision and Microsoft Excel programs. Genes with a twofold induction ratio or higher and signal-to-noise ratios $>3$ in both independent experiments were considered to be induced.

Altogether, the LL-37 treatment induced 96 genes (Table 2, only the first gene of an operon is listed), including several genes that are regulated by the SigW and SigM ECF sigma factors (Huang *et al.*, 1999; Cao *et al.*, 2002a; Thackray & Moir, 2003; Asai *et al.*, 2003). Of the 30 verified promoters of

**Table 1.** Primers used in real-time PCR analyses

| Target gene | Forward primers | Reverse primers |
|---|---|---|
| *bceA* | 5′-cgagcattattttcgctgatga-3′ | 5′-taatagatcggaggccgatttc-3′ |
| *bcrC* (*ywoA*) | 5′-tcgttgcgatacagtgcat-3′ | 5′-tcgtatggtcacttggaaatgaa-3′ |
| *gyr* | 5′-gattattaacctgctggaggtagaaaa-3′ | 5′-aggtaaagctccgcattgaattc-3′ |
| *racX* | 5′-tccgcatcccgcaaaa-3′ | 5′-tggtagatggcggattgga-3′ |
| *radC* | 5′-acaagacggctgtttgaatgc-3′ | 5′-tccccgatcaccaaatgg-3′ |
| *wprA* | 5′-catctctgctccagggtctgata-3′ | 5′-ttccgctcatgtacgtgacatt-3′ |
| *yjbC* | 5′-tgtttgcggaattcgattctt-3′ | 5′-cctgccctcttgcatcctt-3′ |
| *ypuA* | 5′-ccgggactgccgcttta-3′ | 5′-tcctccgagatcgcttcgt-3′ |
| *yuaG* | 5′-ccgatgcagaccgttattctg-3′ | 5′-cgcgagctgtttggcttt-3′ |
| *yvcP* | 5′-tgcgccgcacatatggt-3′ | 5′ -ccgccaagctccactattct-3′ |
| *yvcR* | 5′-gcttgtgccagagcgattatt-3′ | 5′-cgaggtttccgttcggttca-3′ |
| *liaH* (*yvqH*) | 5′-tccgcgcgaattattcaaa-3′ | 5′-cgtctgcgaattctttacgtgta-3′ |
| *liaI* (*yvqI*) | 5′-catctgctcacttccgtttgtc-3′ | 5′-ttccagccgtaatacaccatagc-3′ |
| *yxdJ* | 5′-tgtatatcacgcggctcagaa-3′ | 5′-cgcaccgcttcaatagaaaca-3′ |
| *yxdL* | 5′-ggtcattcataagccgtcactca-3′ | 5′-ttgtcgctttggaatcaaggt-3′ |
| *yxdM* | 5′-tcattgccgtcatgcataca-3′ | 5′-cccgctacatcggaatatcct-3′ |

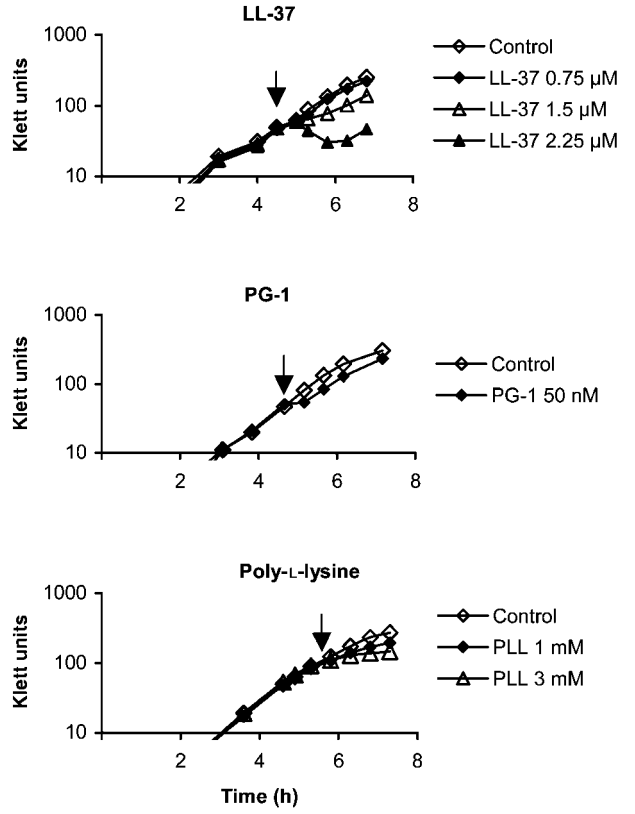



**Fig. 1.** The cationic peptides LL-37, PG-1 and PLL inhibit the growth of *B. subtilis*. Bacteria were grown in BFA minimal medium in shake flasks, and culture densities were measured with a Klett colorimeter. The cationic peptides were added at the cell density of 60 Klett units (arrow).

the SigW regulon (Cao *et al.*, 2002a), only 10 were expressed at elevated levels (greater than twofold) in LL-37-treated cells, with the fold-induction ratios ranging from 2·4 to 14·7, suggesting that LL-37 is a weak SigW inducer. The promoters that are directly regulated by SigM are less well known, but it was observed that 15 candidate promoters of the SigM regulon (Asai *et al.*, 2003), including *sigM* itself, were upregulated by LL-37 (Table 2). Many of the induced genes are involved in extracytoplasmic functions such as synthesis of the cell wall. These genes included *pbpE*, encoding a penicillin-binding protein (4·5-fold induction), *wapA*, encoding a cell wall-associated protein (2·5-fold induction), *murG*, involved in cell wall formation (6·1-fold-induction) and *maf*, required for septum formation (2·5-fold induction). The upregulated genes also included *bcrC* (*ywoA*), which is dependent on several sigma factors and is required for bacitracin resistance (Cao & Helmann, 2002) (4·1-fold induction), and the gene encoding the penicillin-binding protein *ponA* (2·3-fold induction). The most strongly induced gene was *liaI* (*yvqI*) (Mascher *et al.*, 2004), which was induced 58-fold (Table 2); other genes of the *liaIHGFSR* (*yvqIHGFEC*) gene cluster were also upregulated, but to a lesser extent (not shown). *liaI* (*yvqI*) is not known to be dependent on any ECF sigma factor. Furthermore, *yrhH* (14·7-fold), encoding a putative methyltransferase, *yxdL* (22·7-fold) and *yhcG* (14·7-fold), encoding putative ATP-binding components of ABC transporters, and *yoeB* (9·1-fold), encoding a putative exported protein of unknown function, were strongly upregulated.

The second natural peptide, PG-1, induced 58 genes. In a similar manner to LL-37, PG-1 also activated a subset of the genes of the ECF regulons (Table 2). Twelve genes of the SigW regulon, for which there are 30 verified promoters, and five genes predicted to belong to the SigM regulon (Asai *et al.*, 2003) were upregulated. The gene induction pattern resembled that of LL-37, but the induction ratios were lower.

Genes that were induced by both peptides were *araE*, *bcrC* (*ywoA*), *dltB*, *liaI*, *murG*, *pbpE*, *pspA*, *spoOM*, *wprA*, *yceC*, *yeaA*, *yjbC*, *yoeB*, *yqeZ* and *yuaG* (Table 2). Interestingly, the genes of the *yxdLM* operon, *yrhH* and *yhcG*, which were highly induced by LL-37, were not induced by PG-1 (see also below).

PLL was also an ECF inducer and the gene induction pattern resembled that of the other antimicrobial peptides, but characteristic differences were also observed (Table 2). Among 86 upregulated genes, there were 23 and 8 genes that belonged to the SigW and SigM regulons, respectively. Thus, compared to the response to LL-37 (above), it seems that PLL is clearly a stronger activator of the SigW regulon. There were also several genes that were induced at high levels by PLL, but not at all by LL-37 or PG-1, including *csfB* (6·6-fold), *yaaN* (5·1-fold), *yfhL* (5·6-fold), *yocA* (4·5-fold) and *yrzI* (12·4-fold), encoding proteins of unknown function. In addition, genes involved in purine, pyrimidine and ribosomal protein synthesis were strongly induced, a phenomenon not seen with the natural peptides. Furthermore, some genes that were induced at high levels by either or both of the natural peptides were not induced by PLL, notably *liaI* (*yvqI*) and other genes of the *lia* (*yvq*) cluster, *yxdL*, which was strongly induced by LL-37, and *yhcG* and *yoeB*.

Decreased expression of several genes was also observed. However, the experimental setup, short time of exposure to the peptides, and very different degradation rates of mRNAs hampered the interpretation of these results and they were not analysed in this study.

## Cross-talk in signal transduction pathways mediating stress responses induced by a cationic peptide

We also carried out the DNA array analysis with *sigM* and *sigW* knockout mutants using LL-37 for the induction. The gene induction patterns of the sigma mutants and wild-type strain were compared in scatter plots (Fig. 2). The LL-37 treatment elicited a significantly lower number of induced genes in both sigma mutants than in the wild-type. The genes induced in the *sigM* and *sigW* mutants are listed in

**Table 2.** Genes induced by LL-37, PG-1 and PLL

| Gene* | ECF sigma factor regulator† | Fold induction‡ | | |
|---|---|---|---|---|
| | | LL-37 | PG-1 | PLL |
| *abh* | M,W,X | | 2·0 (0·5) | |
| *araE* | | 3·4 (±0·3) | 6·3 (2·1) | 4·4 (±0·9) |
| *bcrC* | M,V,W,X | 4·1 (±0·6) | 2·5 (0·5) | 3·0 (±0·1) |
| *citB* | | 2·4 (±0·3) | | |
| *clpE* | | | 2·5 (0·6) | |
| *codY* | | 2·4 (±0·1) | | |
| *csbD* | | | 2·1 (0·8) | |
| *csfB* | | | | 6·6 (±3·1) |
| *ddlA* | | 2·7 (±0·4) | | |
| *dhbE* | | 2·9 (±0·3) | | |
| *divIC* | M,V,W,X | 2·5 (±0·2) | | |
| *dltA*§ | V,W,X | ¶ | 2·0 (0·5) | ¶ |
| *fur* | | | | 2·6 (±0·3) |
| *glpD* | | 2·4 (±0·4) | | |
| *greA* | M | 2·5 (±0·1) | | |
| *guaC* | | | | 5·8 (±2·4) |
| *iolS* | | 2·3 (±0·2) | | |
| *liaI*§ | | 58·1 (±24·1) | 15·3 (1·1) | |
| *ligA* | M,W | 2·2 (±0·1) | | |
| *maf*§ | M,V,W | 2·5 (±0·1) | | |
| *mrgA* | | | 2·7 (1·3) | |
| *murE* | | | | 2·4 (±0·2) |
| *murG* | M,W,Y | 6·1 (±0·3) | 2·3 (0·5) | |
| *nfrA* | | 3·3 (±0·4) | | |
| *parC* | | 2·3 (±0·1) | | |
| *pbpE* | W | 4·5§ (±0·8) | 3·5§ (0·5) | 2·6 (±0·1) |
| *pbpX* | W,X | | 2·5 (0·3) | |
| *phoA* | M | 4·5 (±1·8) | | |
| *ponA* | M | 2·3 (±0·3) | | 3·1 (±0·1) |
| *pspA* | V,W | 2·7 (±0) | 2·3 (0·8) | 3·5 (±0·3) |
| *pucJ* | | | | 2·6 (±0) |
| *purE* | | | | 4·0 (±0·3) |
| *pyrB*§ | W | | | 18·7 (±3·8) |
| *rplT* | | | | 2·9 (±0·1) |
| *rpmC*§ | V,W | | | 2·2 (±0·2) |
| *ruvB* | | 5·1 (±3·0) | | |
| *sigI* | | | | 6·6 (±1·3) |
| *sigM*§ | M,W | 3·4 (±0·7) | | 4·3 (±1·8) |
| *sigW* | W | | 2·3 (0·2) | 5·1§ (±0·6) |
| *sigX*§ | X | | 2·3 (1·1) | |
| *speD* | | | | 2·2 (±0·1) |
| *spoOM* | W | 4·8 (±1·4) | 2·7 (0·5) | 3·6 (±0·2) |
| *tagA* | | | | 3·8 (±1·3) |
| *tagG* | W | 4·6 (±0·9) | | |
| *trxB* | | 2·5 (±0·5) | | |
| *wapA*§ | W | 2·5 (±0·5) | ¶ | |
| *wprA* | | 2·6 (±0) | 3·5 (0·7) | |
| *yaaK* | | 2·6 (±0·3) | | |
| *yaaN*§ | W | | | 5·1 (±1·1) |
| *ybcH* | | | | 2·5 (±0·1) |
| *ybyB* | | | 2·2 (0·5) | |
| *yceC*§ | M,W,X | 3·5 (±0·1) | 2·1 (0·2) | 3·7 (±0·9) |

**Table 2.** cont.

| Gene* | ECF sigma factor regulator† | Fold induction‡ | | |
|---|---|---|---|---|
| | | LL-37 | PG-1 | PLL |
| *ycnD* | | 2·1 (±0·1) | | |
| *ydbO* | M,V,X | 6·0 (±1·2) | | |
| *ydbS*§ | W | | 2·3 (0·8) | 3·9 (±0·1) |
| *ydiP* | | 3·2 (±0) | | |
| *yeaA* | W | 2·4 (±0·2) | 2·1 (0·6) | 5·4§ (±0·2) |
| *yetG* | | 3·8 (±0·5) | | |
| *yfhK* | | | 5·6 (2·8) | |
| *yfhL*§ | W | | | 5·6 (±0·9) |
| *yfhM* | | | 2·3 (1·5) | |
| *yflT* | | | 2·4 (1·6) | |
| *yhaS* | | 2·7 (±0·4) | | |
| *yhbB* | | 3·5 (±0·6) | | |
| *yhcG* | | 14·7 (±3·4) | | |
| *yhcU* | | 2·3 (±0) | | |
| *yheN* | | | 2·1 (0·5) | |
| *yjbC*§ | M,W,X | 4·4 (±1·2) | 2·9 (0·2) | 3·8 (±0·5) |
| *yjoB* | W | | | 4·2 (±0·5) |
| *yknW* | W | | | 2·9 (±0·5) |
| *yknZ* | W | | 2·1 (0·6) | |
| *ykoJ* | | | 3·3 (0·2) | |
| *ykoK* | | | | 2·9 (±0·6) |
| *ykpC*§ | | | | 6·0 (±0·7) |
| *yktC* | | 2·4 (±0·2) | | |
| *ykvE* | | | | 2·5 (±0) |
| *ykvS* | | 2·6 (±0) | | |
| *yndN* | W | | | 6·0 (±0·3) |
| *yoaF* | W | | | 6·7 (±3·6) |
| *yobJ* | W | | | 2·9 (±0·3) |
| *yocA* | | | | 4·5 (±1·0) |
| *yocH* | W | | | 2·7 (±0·1) |
| *yodT* | | | | 2·2 (±0) |
| *yoeB* | | 9·1 (±1·6) | 3·9 (1·1) | |
| *yojG* | | 2·5 (±0·3) | | |
| *yozO* | W | | | 2·9 (±0·7) |
| *ypbH* | | 2·8 (±0·3) | | |
| *ypuA* | | 5·7 (±0·1) | | 2·9 (±0·8) |
| *ypuD* | | 3·5 (±1·1) | | |
| *ypwA* | | 2·5 (±0) | | |
| *yqeZ*§ | V,W | 2·5 (±0·4) | 3·5 (0·7) | 5·0 (±2·1) |
| *yrbC* | | 3·4 (±0·7) | | |
| *yrhH* | M,W,X | 14·7 (±2·1) | | 4·6 (±0·4) |
| *yrhJ* | M | 3·6 (±0·1) | | |
| *yrkN* | | | | 4·2 (±1·7) |
| *yrrM*§ | V,W | | | 2·9 (±0·1) |
| *yrzG* | | 2·6 (±0·4) | | |
| *yrzI* | | | | 12·4 (±7·1) |
| *ysdA* | | | | 3·1 (±0·6) |
| *ysdC* | | 2·5 (±0·2) | | |
| *ytcJ* | | 2·5 (±0·4) | | |
| *ythP*§ | W | | | 3·8 (±0·8) |
| *ytiP* | | | | 2·3 (±0·1) |
| *ytrF* | | 2·6 (±0·4) | | |

**Table 2.** cont.

| Gene* | ECF sigma factor regulator† | Fold induction‡ | | |
|---|---|---|---|---|
| | | LL-37 | PG-1 | PLL |
| *ytzB* | | 3·5 (±1·1) | | |
| *yuaD* | | 2·3 (±0·2) | | |
| *yuaF*§ | W | ¶ | 5·9 (0·5) | 3·4 (±0·1) |
| *yugP* | | 2·4 (±0·1) | | |
| *yuxN* | | 5·6 (±0·5) | | |
| *yvcR*§ | | 3·5 (±0·6) | | |
| *yvgN* | | 2·8 (±0·2) | | |
| *yvlA*§ | W | | | 3·4 (±0·8) |
| *yvyE* | | | | 4·3 (±2·1) |
| *ywaC* | M,W | | | 4·4 (±0·8) |
| *ywiE* | | | 2·9 (1·1) | |
| *ywjC* | | | 2·1 (0·7) | |
| *ywnF* | | 2·4 (±0·3) | | |
| *ywrE* | W | | 2·4 (0·1) | 7·1 (±2·5) |
| *ywrO* | | 2·6 (±0·4) | | |
| *ywsB* | | | 2·4 (0·7) | |
| *ywzA* | | | 2·1 (0·6) | |
| *ywzC* | | | | 2·1 (±0) |
| *yxdL*§ | | 22·7 (±9·1) | | |
| *yxiE* | M | 2·9 (±0·3) | | |
| *yxjI* | W | 3·4 (±0·2) | | 6·5 (±0·2) |
| *yxkD* | | | | 3·2 (±0·2) |
| *yxkH* | Y | | 2·6 (1·3) | |
| *yyaK* | | | 2·3 (0·1) | |
| *yybD* | | | 2·0 (1·2) | |

*Only the first gene of an operon is listed.

†Evidence suggesting regulation by ECF sigma factors has previously been reported (Huang & Helmann, 1998; Huang *et al.*, 1999; Wiegert *et al.*, 2001; Cao *et al.*, 2002a; Asai *et al.*, 2003; Cao & Helmann, 2004). Underlining indicates a verified promoter (Huang & Helmann, 1998; Cao *et al.*, 2002a; Cao & Helmann, 2004). The ECF sigma factor dependencies of other genes may include indirect effects.

‡The ratio of the expression level of the gene in treated cells to that in untreated cells, as determined by DNA macroarray. Mean of two or three experiments. The standard deviation or range is shown in parentheses.

§Whole operon was induced.

¶Downstream genes were induced.

Table 3. This reduced stress response was not due to decreased stress in the sigma mutants, since the effective concentration of LL-37 causing the growth inhibition was the same in all three strains (see below). In the *sigM* mutant, as expected, the genes of the SigM regulon were not induced. Surprisingly, the array data also showed either that the SigW-regulated genes were not induced or that their induction levels were clearly lower than in the wild-type strain. Analogous results were obtained with the *sigW* mutant. Interestingly, numerous genes which are not known to belong to these two sigma factor regulons were also induced in a SigM- or SigW-dependent manner. These results suggest significant cross-talk between the SigM and SigW regulons, and some other regulon(s) responding to LL-37. Furthermore, a significant observation was the clearly higher level of expression of several genes in the *sigW* mutant compared to that of the wild-type (Fig. 2b). This set included (Table 3) genes encoding endo-1,4-β-glucanase (*bglC*; 4·6-fold), a protein synthesizing α-1,4-glucan using ADP-glucose (*glgA*; 5·1-fold) and 6-phospho-α-glucosidase (*glvA*/*malA*; 3·9-fold).

In order to study the overlap between the stress regulons further, we determined the expression levels of some of the induced genes by real-time RT-PCR. The *liaI* and *yxdL* genes were chosen due to their strong induction with LL-37. The *yuaG* gene (in the *yuaFGI* operon) was induced most strongly by PG-1. It encodes a putative flotillin known to belong to the SigW regulon (Huang *et al.*, 1999; Cao *et al.*, 2002a). Other chosen genes included *racX* (in an operon

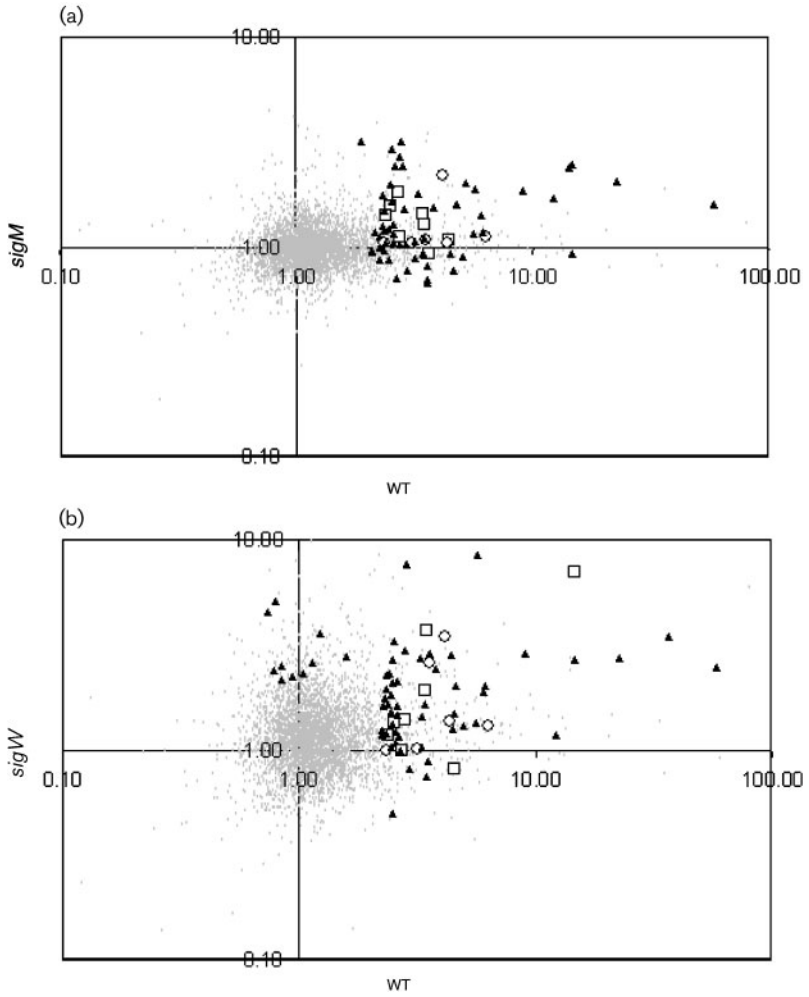



**Fig. 2.** Comparison of the induction ratios of the wild-type strain and the *sigM* (a) and s*igW* (b) mutants treated with LL-37. The genes induced in the wild-type and/or a sigma mutant by LL-37 are highlighted (closed triangle, open circle or square). The genes belonging to the SigM or SigW regulons are marked with open circles and squares, respectively. The induction ratios of other genes are marked with grey circles and include some high induction ratios due to the use of non-filtered data in this graphical comparison; the filtering (see Methods) of data eliminated these high induction ratios. WT, wild-type.

with *pbpE*), a SigW-dependent gene (Cao *et al.*, 2002a), and *yjbC* and *bcrC* (*ywoA*), which belong to the SigM regulon (Cao & Helmann, 2002; Ohki *et al.*, 2003a). The *yjbC* gene is also regulated by SigW and SigX (Cao *et al.*, 2002a; Ohki *et al.*, 2003a; Thackray & Moir, 2003). Furthermore, the expression of *radC* (in an operon with *maf*), which may be regulated by SigM (Asai *et al.*, 2003), and of *wprA* and *ypuA*, which may be expressed independently of the ECF sigma factors (Table 2), was determined. The RT-PCR analysis was carried out with the wild-type strain and the *sigM* and *sigW* mutants. A *sigM sigW* double mutant was also used to analyse the expression of some genes. Bacteria were treated in a similar manner to that employed in the array experiments with LL-37 or PG-1. Since the array analysis revealed that the induction of *radC* and *ypuA* with PG-1 was low, their induction with this peptide was not determined. Samples were collected for analysis at two different time points, 10 and 20 min after the addition of the antimicrobial peptides. All mRNA measurements were performed two to four times.

In the wild-type strain treated with LL-37, *liaI* and *yxdL* were the most highly induced genes (Table 4), as was also the case in the array analysis. Higher induction ratios were seen in the 10 min samples than in the 20 min samples, indicating that the induction was fast and transient. As shown in Fig. 3, expression was dependent on the dosage of LL-37, as demonstrated with the *liaI* gene (Fig. 3a), and decreased (also that of *liaH*) from the maximal level (10 min time point) back to the uninduced level in about 2 h (Fig. 3b). Consistent with the induction of the *liaIHGFSR* gene cluster, the LiaH protein appeared in the proteome of cytoplasmic proteins, as demonstrated by two-dimensional gel electrophoresis and spot identification by matrix-associated laser desorption ionization–time of flight mass spectrometry (MALDI-TOF) (data not shown). The other genes were induced to a lesser extent, and in only half of them was the induction transient (Table 4). The normalized mRNA levels (not shown) indicated that *liaI*, *yxdL* and *yuaG* were expressed at a very low level in non-treated cells (basal expression level). The basal expression level of *wprA*, *yjbC*, *ypuA* and *radC* was fairly high, and *bcrC* (*ywoA*) and *racX* were expressed at intermediate levels. The genes that were expressed at a low level in non-treated cells exhibited the strongest induction in peptide-treated cells. Normalized mRNA levels of the genes varied approximately

**Table 3.** Induction of gene expression in cells of the *sigW* and *sigM* mutants treated with LL-37

| Gene | Fold induction* | | |
|---|---|---|---|
| | *sigW* | *sigM* | WT† |
| *bcrC* | 3·5 (±0·2) | | 4·1 (±0·6) |
| *bglC* | 4·6 (±1·2) | | |
| *cydC* | | 2·4 (±0·2) | |
| *dltB* | 2·3 (±0·2) | 3·2 (±0·4) | 2·8 (±0·4) |
| *dltC* | | 2·9 (±0·4) | 2·5 (±0·4) |
| *ftsY* | 2·3 (±0) | | |
| *glgA* | 5·1 (±1·5) | | |
| *glvA* | 3·9 (±1·2) | | |
| *liaI* | 2·4 (±0·4) | | 58·1 (±24) |
| *minC* | 3·5 (±0·1) | | 2·3 (±0·2) |
| *qoxC* | 2·5 (±0·5) | | |
| *sigX* | | 2·4 (±0·2) | 2·6 (±0·6) |
| *wapA* | | 2·9 (±0·1) | 2·6 (±0) |
| *yceC* | 3·7 (±0·5) | | 3·5 (±0·1) |
| *yfiJ* | 3·6 (±1·4) | | |
| *yhcH* | 2·4 (±0·1) | | 4·6 (±1·2) |
| *yisV* | 2·4 (±0) | | |
| *ykfA* | 2·1 (±0) | | |
| *ypbH* | 5·8 (±3·8) | | 2·8 (±0·3) |
| *ypuA* | 8·4 (±1·9) | | 5·7 (±0·1) |
| *yrhH* | 7·0 (±3·3) | | 14·7 (±2·1) |
| *yrhJ* | 2·9 (±0·7) | | 3·6 (±0·1) |
| *yrvP* | 2·6 (±0·1) | | |
| *yrzG* | | 2·8 (±0·4) | 2·6 (±0·4) |
| *ytrF* | 2·8 (±0·5) | | 4·4 (±0·7) |
| *yvgN* | 7·6 (±4·7) | | 2·8 (±0·2) |
| *ywaC* | 4·0 (±0·1) | | 7·4 (±1·8) |
| *ywdA* | | 2·0 (±0) | |
| *yxdL* | 2·7 (±0·5) | | 22·7 (±9·1) |
| *yxeA* | 3·5 (±0·4) | | 36·4 (±2·1) |
| *yxiG* | 3·4 (±0·1) | 2·7 (±0) | 2·4 (±0·2) |
| *yxjF* | 2·2 (±0·2) | | |
| *yxkH* | 3·0 (±0·3) | 2·4 (±0·2) | |
| *yxxG* | 4·5 (±0·9) | 3·4 (±0·8) | 3·6 (±0·6) |
| *yxzC* | 3·5 (±0·2) | 3·2 (±0·2) | 3·6 (±0·1) |
| *yxzG* | 2·5 (±0·2) | | 2·9 (±1·0) |
| *yyaK* | | 3·2 (±1·1) | |

*The ratio of the expression level of the gene in treated cells to that in untreated cells, as determined by DNA macroarray. Mean of two experiments. The range is shown in parentheses.

†The induction ratios of all genes (or the first genes of operons) induced in a wild-type (WT) strain (LL-37 treatment) are shown in Table 2.

twofold from one experiment to another and between non-treated wild-type and ECF sigma mutant cells (not shown). Considerable experimental variation was especially observed in the induction ratios of the *liaI* and *yxdL* genes (Table 4). The RT-PCR displayed clearly higher induction ratios than the DNA array, but there was a good overall consistency of the induction pattern in these two types of assay.

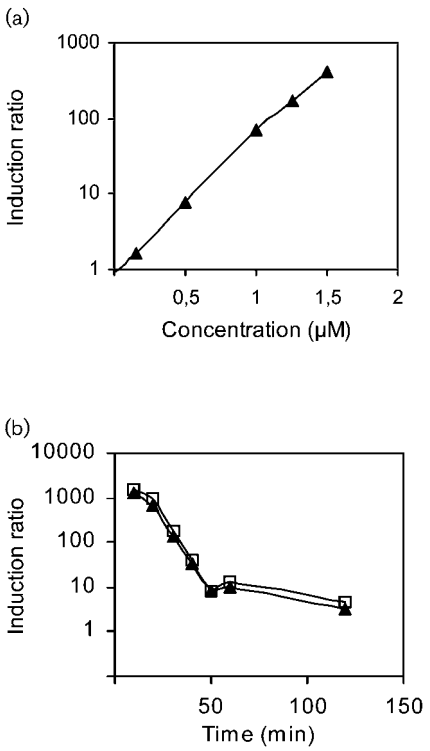
The *liaI* gene, which was induced 1530-fold in the wild-type (10 min time point) with LL-37, was also upregulated in the *sigM* and *sigW* mutants, but in both of them the induction was clearly lower, 198- and 287-fold, respectively (Table 4). In DNA arrays, the difference in the induction ratios was more dramatic, 58-fold in the wild-type and twofold in the mutants. The other highly induced gene, *yxdL*, was also upregulated significantly less in the *sigM* (248-fold) and *sigW* (347-fold) mutants compared to the wild-type strain (704-fold), consistent with the array results above. Only a minor additive effect on *liaI* and *yxdL* expression was observed in the double mutant (Table 4). The *yuaG* (*yuaFGI*) gene was induced 18-fold (10 min time point), whereas no induction was seen in the *sigW* mutant, consistent with its previously verified SigW dependency (Cao *et al.*, 2002a) (see also below). The induction of *yuaG* was also reduced in the *sigM* mutant (sevenfold at the 10 min time point), suggesting moderate SigM dependency. The *yjbC* and *bcrC* (*ywoA*) genes were induced in the wild-type strain 6–11-fold, whereas in the *sigM sigW* double mutant almost no induction was seen (Table 4), indicating that these genes are induced in an ECF-dependent manner. However, the inactivation of single ECF sigma factors reduced the induction ratios only moderately or not at all. LL-37 induced *yjbC* expression three- to fivefold in the *sigM* mutant and six- to ninefold in the *sigW* mutant. The induction ratios of *bcrC* were 3–6 (*sigM*) and 6–9 (*sigW*). *racX* induction was affected by both sigma mutations (see also the effects of PG-1 and Triton X-100 on *racX* expression below). These results suggest that both SigM and SigW regulate either directly or indirectly the *yjbC*, *bcrC* (*ywoA*) and *racX* genes. LL-37 upregulated the expression of *radC*, *wprA* and *ypuA* four- to 12-fold in the wild-type. Reduced induction ratios of *radC* and *ypuA* in the *sigM* mutant suggest that their induction by LL-37 is at least partially mediated by SigM (Table 4). The *wprA* gene exhibited induction patterns that rather suggest the independence of the sigma factors (see also the effects of PG-1 and Triton X-100 on *wprA* expression below).

The induction ratios of all genes studied were significantly lower in PG-1-treated cells than in LL-37-treated cells (Table 5), suggesting that the stress caused by the PG-1 treatment was less severe; for example, the induction ratio of *liaI* with LL-37 was several-fold higher than with PG-1. Despite the lower induction level in PG-1-treated cells, the *sigM* and *sigW* mutations reduced the induction of *liaI* as in LL-37-treated cells (Table 5). The concentration of PG-1 used induced the *yjbC* and *bcrC* (*ywoA*) genes very weakly in all three strains. PG-1 treatment induced *yuaG* expression in a similar manner to LL-37 treatment, and the strong SigW dependency and moderate SigM dependency of *yuaG* were also observed with PG-1 (Table 5). In the wild-type, *racX* was induced four- to ninefold, whereas no induction was observed in the *sigW* mutant, suggesting SigW-dependent regulation of *racX*. In the *sigM* mutant, *racX* was induced two- to sixfold by PG-1. The inactivation of the sigma factors did not impair the induction of *wprA*,

**Table 4.** The dependency of a set of LL-37-induced genes on SigM and SigW

| Gene | Fold induction* | | | | | | | | | | | Function of encoded protein |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | WT | | | Mutation† | | | | | | | | |
| | | | | *sigW* | | | *sigM* | | | *sigW* | sig*M* | |
| | 10 min | 20 min | Array | 10 min | 20 min | Array | 10 min | 20 min | Array | 10 min | 20 min | |
| *bcrC* | 9·9 (0·9) | 6·0 (2·5) | 4·1 | 9·2 (5·2) | 6·1 (2·7) | 3·5 | 3·2 (0·8) | 6·1 (0·9) | 5·9 | 1·8 (±0·2) | 2·1 (±0·2) | Bacteriocin transport permease |
| *racX* | 6·0 (2·5) | 6·9 (3·5) | 5·0 | 1·5 (0·5) | 2·4 (0·6) | 1 | 3·2 (1·6) | 2·8 (0·7) | 1·6 | 1·4 (±0·1) | 2·3 (±0·1) | Amino acid racemase |
| *radC* | 6·3 (±1·1) | 3·9 (±0·8) | 2·4 | 3·4 (±3·4) | 2·8 (±0·2) | 0·9 | 1·3 (±0·1) | 1·5‡ | 1·8 | | | DNA repair protein |
| *wprA* | 6·4 (1·8) | 5·6 (2·1) | 2·6 | 3·2 (1·2) | 2·9 (0·2) | 1·7 | 6·1 (2·5) | 6·4 (1·1) | 3·6 | 2·4 (±0·1) | 3·4 (±0·2) | Cell wall-associated protein precursor |
| *yjbC* | 11·5 (2·9) | 9·1 (3·0) | 4·4 | 9·0 (7·2) | 6·4 (4·7) | 1·4 | 3·0 (0·8) | 4·7 (1·7) | 1 | 1·1 (±0) | 1·4 (±0·29) | Unknown |
| *ypuA* | 10·4 (±0·2) | 12 (±4·4) | 5·7 | 11·0 (±2·6) | 11·6 (±0·8) | 8·4 | 2·6 (±0·5) | 4·2‡ | 2·6 | | | Unknown |
| *yuaG* | 17·7 (±4·5) | 7·4 (±0) | 2·7 | 1·3 (±0·4) | 1·1 (±0·1) | 1 | 6·9 (±0·1) | 3·5‡ | 2·1 | | | Similar to flotillin 1 |
| *liaI* (*yvqI*) | 1530 (860) | 610 (560) | 58·1 | 287 (220) | 51 (45) | 2·4 | 198 (200) | 54 (69) | 1·7 | 106 (±41) | 37 (±16) | Unknown |
| *yxdL* | 704 (113) | 338 (63) | 22·7 | 347 (219) | 126 (90) | 2·7 | 248 (48) | 49 (15) | 2·3 | 123 (±17) | 29 (±5·2) | Similar to ABC transporter |

*The ratio of the expression level of the gene in LL-37-treated cells to that in untreated cells, as determined by RT-PCR. The ratios from the DNA array analyses, if determined (Table 2), are also shown. The standard deviation or range is shown in parentheses. WT, wild-type.
†The *sigW*::*neo* (constructed in the laboratory of J. D. Helmann) and *sigM*::pMUTIN4 (Horsburgh & Moir, 1999) knockout mutations. The *sigW sigM* double mutant contains both of them.
‡Only one measurement.




consistent with an induction mechanism that is independent of SigW and SigM.

## Triton X-100 induces sigma regulons, but in a pattern different from that of antimicrobial peptides

In order to find out whether the above genes are also induced by a membrane-disrupting agent with no presumed specificity, we treated cells with 0·005 % Triton X-100 and determined expression levels of the genes in the wild-type strain and the ECF sigma factor mutants by real-time RT-PCR. The effects of cationic peptides on membranes may be

**Fig. 3.** Dependence of *lial* expression on the concentration of LL-37 and kinetics of LL-37-induced expression of *lial* and *liaH*. Bacteria were grown in BFA minimal medium. (a) The induction of *lial* in cells stressed with various concentrations of LL-37. (b) LL-37 (1·5 μM) was added at the cell density of 60 Klett units and the expression of *lial* (closed triangle) and *liaH* (open square) was determined by RT-PCR at various time points of the LL-37 treatment. The induction ratio is fold induction in LL-37-treated cells compared to that in non-treated cells.

**Table 5.** Induction of a set of genes by PG-1 and their dependence on SigM and SigW

| Gene | Fold induction* | | | | | | | Function of encoded protein |
|---|---|---|---|---|---|---|---|---|
| | WT | | | Mutation† | | | | |
| | | | | *sigW* | | *sigM* | | |
| | 10 min | 20 min | Array | 10 min | 20 min | 10 min | 20 min | |
| *bcrC* | 2·0 (±0·1) | 3·3 (±0·2) | 2·5 | 1·7 (±0·1) | 2·2 (±1·0) | 1·2 (±0·1) | 5·3 (±0·2) | Bacteriocin transport permease |
| *racX* | 4·3 (±1·1) | 8·7 (±0·1) | 3·8 | 1·1 (±0) | 1·3 (±0·1) | 2·5 (±0·1) | 5·9 (±0·7) | Amino acid racemase |
| *wprA* | 1·4 (±0·1) | 3·9 (±0·7) | 3·5 | 1·9 (±0·1) | 3·8 (±0·5) | 2·3 (±0·2) | 4·1 (±0·3) | Cell wall-associated protein precursor |
| *yjbC* | 1·3 (±0·3) | 2·6 (±0·4) | 2·9 | 1·6 (±0·1) | 1·8 (±0·2) | 2·6 (±0·2) | 2·3 (±0·2) | Unknown |
| *yuaG* | 17·1 (±0·5) | 9·7 (±0·4) | 6·8 | 0·5 (±0·2) | 0·5 (±0·1) | 6·4 (±0·1) | 4·3 (±0·9) | Similar to flotillin 1 |
| *liaI* (*yvqI*) | 328 (±34) | 258 (±36) | 15·3 | 118 (±2) | 59 (±11) | 13·3 (±3·3) | 13·2 (±2·4) | Unknown |
| *yxdL* | 1·5 (±0·1) | 1·4 (±0) | 1·4 | 0·9 (±0·1) | 0·9 (±0·1) | 1·1 (±0·1) | 1·1 (±0·2) | Similar to ABC transporter |

*The ratio of the expression level of the gene in PG-1-treated cells to that in untreated cells, as determined by RT-PCR. The ratios from the DNA array analyses, if determined (Table 2), are also shown. The range is shown in parentheses. WT, wild-type.
†The *sigW*::*neo* (constructed in the laboratory of J. D. Helmann) and *sigM*::pMUTIN4 (Horsburgh & Moir, 1999) knockout mutations.

somewhat different from those of detergents (Henzler Wildman *et al.*, 2003), although the opposite view has also been put forward (Oren *et al.*, 1999).

A similar induction of *liaI* expression (790-fold at the 10 min time point) and slightly reduced induction ratios in the *sigM* and *sigW* mutants, as with LL-37, were observed (Table 6). The strong dependency of *yuaG* on SigW was also demonstrated with the detergent, but the moderate SigM dependency was not observed. The induction of three other genes (*radC*, *ypuA* and *bcrC*) was also partially dependent on both sigma factors. *radC* was expressed in the wild-type strain at two- to fivefold, *ypuA* at two- to threefold and *bcrC* (*ywoA*) at threefold higher levels than in the sigma mutants (Table 6). In the wild-type strain and *sigM* mutant, a clearly stronger induction of *racX* was observed when cells were treated with Triton X-100 (19–61-fold) than when they were treated with the antimicrobial peptides (four- to ninefold). In a similar manner to that observed with PG-1, no induction was seen in the *sigW* mutant, consistent with the SigW dependency of *racX*. The sigma mutations did not impair the induction of *yjbC* in Triton X-100-treated cells, in contrast to LL-37-treated cells. Furthermore, the *wprA* gene was not induced by Triton-X-100.

**Table 6.** Induction ratios in cells treated with Triton X-100

| Gene | Fold induction* | | | | | | Function of encoded protein |
|---|---|---|---|---|---|---|---|
| | WT | | Mutation† | | | | |
| | | | *sigW* | | *sigM* | | |
| | 10 min | 20 min | 10 min | 20 min | 10 min | 20 min | |
| *bcrC* | 6·3 (±1·4) | 9·7 (±2·8) | 2·4 (±0·2) | 2·7 (±0·4) | 1·9 (±0) | 2·8 (±0·1) | Bacteriocin transport permease |
| *racX* | 40·4 (±1·6) | 61·4 (±9·4) | 1·0 (±0·1) | 0·8 (±0) | 19 (±1·5) | 26·3 (±3·5) | Amino acid racemase |
| *radC* | 3·9 (±0·3) | 5·0 (±0·5) | 1·6 (±0) | 1·6 (±0·1) | 1·2 (±0·3) | 0·8 (±0·1) | DNA repair protein |
| *wprA* | 1·3 (±0·1) | 2·0 (±0·2) | 1·4 (±0·2) | 1·4 (±0·1) | 1·9 (±0·1) | 2·6 (±0·1) | Cell wall-associated protein precursor |
| *yjbC* | 4·6 (±0·9) | 5·1 (±0·3) | 5·8 (±0·1) | 7·2 (±0·7) | 11·9 (±0·2) | 5·1 (±0·2) | Unknown |
| *ypuA* | 6·9 (±0·6) | 10·1 (±0·4) | 2·1 (±0·1) | 3·5 (±0·1) | 3·7 (±0·3) | 2·9 (±0·1) | Unknown |
| *yuaG* | 42·1 (±3·6) | 19·4 (±8·4) | 1·4 (±0·5) | 1·2 (±0·2) | 37·5 (±2·6) | 12·8 (±0·1) | Similar to flotillin 1 |
| *liaI* (*yvqI*) | 790 (±276) | 441 (±58) | 347 (±48) | 192 (±44) | 375 (±77) | 227 (±16) | Unknown |
| *yxdL* | 1·0 (±0·1) | 1·6 (±0·2) | 1·4 (±0·2) | 0·6 (±0) | 1·5 (±0·1) | 1·3 (±0·1) | Similar to ABC transporter |

*The ratio of the expression level of the gene in cells treated with Triton X-100 to that in untreated cells, as determined by RT-PCR. The range is shown in parentheses. WT, wild-type.
†The *sigW*::*neo* (constructed in the laboratory of J. D. Helmann) and *sigM*::pMUTIN4 (Horsburgh & Moir, 1999) knockout mutations.

### Inactivation of SigM and SigW does not increase the sensitivity of *B. subtilis* to antimicrobial peptides

The sensitivity of *sigW* and *sigM* mutants and the *sigM sigW* double mutant to LL-37, PG-1 and PLL was tested. Growth experiments were performed in shake-flask cultures in the same conditions as in the array experiments. The growth was also studied with several peptide concentrations with Bioscreen. No significant difference in the sensitivity of the *sigW*, *sigM* or *sigM sigW* mutants to the antimicrobial peptides was detected and the growth arrest caused by the antimicrobial peptides was similar to that of the wild-type. However, it was obvious that the *sigW* and *sigM sigW* mutants grew slower than the wild-type (in the absence of peptide) in the BFA minimal medium containing 100 mM NaCl, indicating the importance of the SigW regulon in these conditions (data not shown). The growth of the *sigM* mutant resembled that of the wild-type.

### *yxdL* gene expression is specifically induced by LL-37

A highly interesting observation was that the *yxdL* gene, which was strongly induced by LL-37 (704-fold), was not induced by PG-1 (Table 5). The lack of induction was seen in both the DNA array and RT-PCR analyses, and it was true for both wild-type and *sigM* and *sigW* mutant cells. The *yxdL* gene was not induced by Triton X-100 either (Table 6). These results suggest that there is a strong specificity in the induction mechanism of *yxdL*.

### LL-37 causes upregulation of three paralogous ABC-transporter genes via TCS-mediated signalling dedicated to the regulation of transporter expression

The *yxdL* gene encodes the putative ATP-binding component of an ABC transporter of unknown function. The downstream gene *yxdM*, which most probably forms an operon with *yxdL*, encodes the permease component of the ABC transporter. Immediately upstream from *yxdLM* there are the *yxdJ* and *yxdK* genes, which encode the components of a TCS of unknown function (see the organization of the gene cluster in Fig. 4). The *yxeA* gene, which encodes a conserved protein of unknown function, is located downstream from the *yxdM* gene and most probably belongs to the same operon as *yxdLM* (not shown in Fig. 4). The DNA array data also revealed that LL-37 strongly induced the *yxeA* gene (36-fold) in a similar manner to *yxdLM*, consistent with the operon organization. A sequence similarity search revealed that *yxdL* and *yxdM* are highly homologous with the corresponding genes of two other ABC transporters of *B. subtilis*, BceAB (formerly YtsCD) and YvcRS (Fig. 4; see also Joseph *et al.*, 2002; Mascher *et al.*, 2003; Ohki *et al.*, 2003b). Interestingly, genes encoding TCSs are also located in the immediate upstream regions of the *bceAB* and *yvcRS* genes in a pattern similar to that of the *yxdLM* region. It has been shown that the BceRS TCS is able to sense extracellular bacitracin and induce the expression of the BceAB ABC transporter, which confers resistance to bacitracin (Mascher *et al.*, 2003; Ohki *et al.*, 2003b). The YxdLM ABC transporter as well as the YxdJK TCS exhibit homology with the corresponding proteins of the Yvc and Bce systems. The homology is highest between the ATP-binding components of the ABC transporters (about 50 % identity), and, in the following order, is less between the response regulators of TCS, the sensor kinases of TCS and the permease components of the ABC transporters (Fig. 4). The Yxd proteins exhibit slightly higher similarity to the Yvc proteins than to the Bce proteins.

The DNA array analysis showed that the *yvcR* and *yvcS* genes were upregulated 3·5- and sixfold in LL-37-treated cells, respectively, suggesting that this antimicrobial peptide may affect not only *yxdL* expression but also its paralogues. The regulation of the three paralogous ABC transporter genes was studied by determining their expression in the wild-type strain and mutants of the upstream histidine kinase genes (*yxdK*, *yvcQ* and *bceS*) treated with LL-37 using real-time RT-PCR. In the wild-type strain, the *yxdM* gene was induced strongly (about 300-fold at the 10 min time point) in a similar manner to the *yxdL* gene (Table 7), further indicating that these genes form an operon (together with *yxeA*). Since no induction of the upstream *yxdJ* gene was observed, the TCS genes must belong to a different transcriptional unit than *yxdLM*, in a manner similar to that by which the *bceRS* genes are transcribed separately from *bceAB* (Joseph *et al.*, 2002; Ohki *et al.*, 2003b). In the *yxdK* mutant, hardly any induction of *yxdL* and *yxdM* was seen, indicating that the YxdJK TCS regulates the expression of the *yxdLM* operon (Table 7). The induction of *yxdL* was also slightly reduced (about 25 %) in both the *yvcQ* and the *bceS*

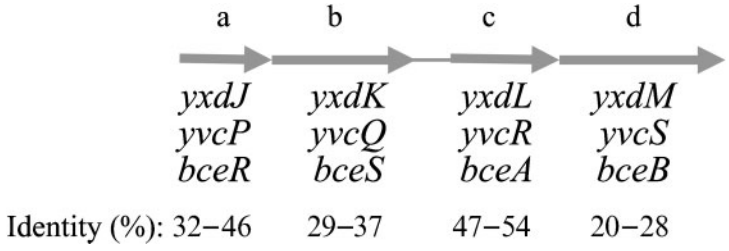



**Fig. 4.** Organization of three pairs of genes encoding homologous ABC transporters and in their immediate upstream region three similar pairs of genes encoding homologous TCSs. The identity values of the amino acid sequences of the deduced protein products are indicated. a, Response regulator of TCS; b, sensor histidine kinase of TCS; c, ATP-binding component of ABC transporter; d, permease component of ABC transporter.

**Table 7.** Expression regulation and cross-regulation of the *yxdLM* operon and its homologues *yvcRS* and *bceAB* via the YxdK, YvcQ and BceS TCSs

| Gene | Fold induction* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | WT | | Mutation† | | | | | |
| | | | *yxdK* | | *yvcQ* | | *bceS* | |
| | 10 min | 20 min | 10 min | 20 min | 10 min | 20 min | 10 min | 20 min |
| *bceA* | 6·1 (0·8) | 3·8 (1·0) | 8·1 (3·4) | 3·4 (0·8) | 7·0 (3·0) | 3·6 (1·0) | 3·3 (±0·4) | 0·6 (±0·1) |
| *yvcP* | 0·8 (0·2) | 0·8 (0·2) | | | | | | |
| *yvcR* | 5·6 (0·5) | 7·8 (4·6) | 3·4 (1·2) | 1·4 (0·4) | 2·2 (0·6) | 3·2 (0·5) | 7·2 (±0·3) | 3·2 (±0·1) |
| *yxdJ* | 1·2 (0·4) | 0·8 (0·3) | | | | | | |
| *yxdL* | 523 (228) | 197 (12) | 1·5 (±0·3) | 1·8 (±0·1) | 328 (142) | 148 (53) | 324 (±67) | 148 (±34) |
| *yxdM* | 320 (37) | 239 (47) | 1·4 (0·5) | 2·6 (1·0) | | | | |

*The ratio of the expression level of the gene in LL-37-treated cells to that in untreated cells, as determined by RT-PCR. The standard deviation or range is shown in parentheses. WT, wild-type.
†Knockout mutations constructed with pMUTIN4 (Vagner *et al.*, 1998).

mutant, suggesting cross-regulation between the systems; in other words, the paralogous TCSs also regulate the promoters of the non-cognate paralogous ABC-transporter genes. The *yvcR* gene was induced by LL-37, but only about fivefold, consistent with the DNA array result for *yvcRS* induction. The inactivation of the upstream sensor kinase gene *yvcQ* only partially abolished the induction of *yvcR* (2·2-fold induction at the 10 min time point). Similar partially abolished induction was also seen in the *yxdK* mutant (3·4-fold), consistent with cross-regulation (Table 7). In the *bceS* mutant, the induction of *yvcR* was comparable to that of the wild-type. LL-37 did not induce *yvcP* expression, indicating that *yvcPQ* and *yvcRS* are different transcriptional units. The DNA array results suggested that the *bceAB* operon is not induced by LL-37. The RT-PCR analysis, however, revealed that *bceA* is also induced about sixfold in LL-37-treated cells (Table 7). The *bceS* mutation partially abolished the induction (3·3-fold), but the *yvcQ* and *yxdK* mutations did not affect it.

In order to find out whether or not the YxdLM or YvcRS ABC transporters have a role in peptide resistance, the sensitivity of *yxdL* and *yxdL yvcR* mutants to LL-37 was tested. Neither the single mutant nor the double mutant showed increased sensitivity to LL-37.

## DISCUSSION

In this study, we carried out genome-wide transcription analyses of the stress responses of *B. subtilis* to the cationic antimicrobial peptides LL-37 and PG-1, which are natural peptides, and PLL, a synthetic peptide. The responses to each peptide were highly complex, including activation of several signal transduction pathways.

The antimicrobial peptides induced expression of 96 (LL-37), 58 (PG-1) and 86 (PLL) genes in *B. subtilis*. In this complex response, some patterns were recognized. A prominent feature was a high proportion of induced genes belonging to the SigW and SigM ECF-type sigma factor regulons. However, only subsets of these sigma factor regulon genes were induced by the antimicrobial peptides. The non-amphipathic PLL was the most effective peptide in activating the SigW regulon, as evidenced by the induction of 23 out of the 30 verified promoters of the regulon [see Cao *et al.* (2002a) for the SigW regulon]. The amphipathic peptides LL-37 and PG-1 upregulated only about one-third of the 30 SigW-regulated genes. In a similar manner, only subsets of putative SigM-regulated promoters were induced in peptide-treated cells. These results suggest that SigW and SigM are involved in the stress responses to the antimicrobial peptides. However, the high numbers of induced genes that are expressed independently of SigW and SigM suggest that probably several other signal transduction pathways and regulators also mediate the stress responses.

It has been shown that the ECF sigma factor regulons are partly overlapping (Cao *et al.*, 2002b; Huang *et al.*, 1998). Nevertheless, the effect of *sigW* or *sigM* mutation on the number of induced genes in cells treated with LL-37 was striking. Not only the genes belonging to the mutated sigma regulon but also those under the control of other ECF sigma factors and those expressed independently of these sigma factors were poorly induced after peptide treatment. This phenomenon may be partly due to the increased basal expression level of several genes in the *sigW* and *sigM* mutants. Consequently, the additional stress of cationic peptides may not have caused further induction. Yet this alone does not explain why so few genes were induced in the sigma factor mutants, since the basal expression level was elevated in the sigma mutants only in the case of about 30 % of the genes induced in the wild-type. The RT-PCR analysis revealed decreased induction ratios for *bcrC*, *liaI*, *radC*, *racX*, *ypuA* and *yxdL* in both the sigma

mutants. However, it is apparent that not all the affected genes are directly regulated by the sigma factors. It has been shown that *liaI* is regulated by the LiaRS (YvqCE) TCS (Mascher *et al.*, 2003; H.-L. Hyyryläinen and others, unpublished results). The effects of ECF sigma factor mutations on *liaI* expression in cells treated with alkaline (Wiegert *et al.*, 2001) or cationic antimicrobial peptides (this study) are most probably indirect. It is possible that the inactivation of one ECF sigma factor results in disturbance of the sensory function of other membrane-associated stress sensors. The similarity of the lethal doses of the cationic peptides in the sigma factor mutants and the wild-type, however, suggests that the degree of stress in these strains was the same. These results suggest that the stress response to cationic antimicrobial peptides is very complex. The functional overlap of several sigma factors and other types of regulators may also explain why the inactivation of SigW, SigM or both of them did not make cells sensitive to antimicrobial peptides.

PLL is expected to interact with the negatively charged cell wall and head groups of the membrane phospholipids (carpet or detergent-like mechanism; Yeaman & Yount, 2003), but may not penetrate deeper into the membrane interior. LL-37 and PG-1 penetrate into the membrane and disturb its integrity by forming pores (Henzler Wildman *et al.*, 2003; Oren *et al.*, 1999; Yang *et al.*, 2000). We hypothesize that SigW-regulated promoters are activated by antimicrobial peptides by the interaction of the latter with the cell membrane surface and the cell wall, rather than by deeper effects inside the membrane. This conclusion is consistent with the strong induction of the SigW regulon by cell wall antibiotics (Cao *et al.*, 2002b).

The *araE*, *bcrC* (*ywoA*), *dltB*, *pbpE*, *pspA* (*ydjF*), *yceC*, *spo0M*, *yeaA*, *yjbC*, *yqeZ* and *yuaG* genes were induced by all three peptide treatments. Each of these genes belongs to at least one ECF sigma factor regulon (Table 2). Some of these genes are involved in interactions with antimicrobial compounds interfering with the cell wall or membrane: *pbpE* encodes a penicillin-binding protein, *yceC* is similar to the tellurium resistance proteins, and *bcrC* (*ywoA*) encodes a bacitracin permease (Cao & Helmann, 2002; Podlesek *et al.*, 1995). The *dlt* operon including *dltB* is involved in the D-alanine esterification of lipoteichoic and wall teichoic acids (Perego *et al.*, 1995), which increases bacterial resistance to cationic antimicrobial peptides (Peschel *et al.*, 1999; Cao & Helmann, 2004).

The DNA array and real-time RT-PCR analyses revealed that not only the ECF sigma factors but also TCSs have a major role in sensing antimicrobial peptides. Most importantly, LL-37 induced the genes of three ABC-type transporters; *yxdLM* was induced strongly (about 700-fold) and its close homologues *yvcRS* and *bceAB* were induced moderately (about sixfold). All these ABC-transporter genes are regulated by TCSs. The TCSs are encoded by genes in the immediate upstream regions of the ABC transporter genes, as evidenced by the lack/decrease of the induction in TCS mutants (Mascher *et al.*, 2003; Ohki *et al.*, 2003b; this study) or demonstrated by primer extension and DNase protection experiments (Pascale *et al.*, 2004). In this study, the results suggested some low-level cross-talk between the three TCSs.

It has been shown that the expression of *bceAB* is induced more than 200-fold by bacitracin via BceRS TCS-mediated signalling (Mascher *et al.*, 2003) and that the BceAB ABC transporter has a role in bacitracin resistance (Ohki *et al.*, 2003b). In contrast, the YxdLM and YvcRS ABC transporters are of unknown function. These TCSs may be involved in sensing conditions inside the cell membrane (Mascher *et al.*, 2003).

This study demonstrates for the first time an activator of the YxdJK TCS: LL-37. Our results also indicate that Triton X-100 and PLL do not activate YxdJK, suggesting distinctly different modes of action for LL-37 and detergents/detergent-like molecules, in contrast to what has been claimed (Oren *et al.*, 1999). LL-37 is most probably a pore-forming peptide (Henzler Wildman *et al.*, 2003), and the penetration of this amphipathic molecule into the membrane is probably crucial for the activation of the YxdJK TCS. The very short extracytoplasmic loop of the YxdK sensor is consistent with the conclusion that YxdJK senses signals inside the membrane, possibly by direct interaction with LL-37, and not signals on the membrane surface or cell wall, or membrane disturbance as such.

PG-1 did not activate YxdJK either, being a pore-forming peptide (Yang *et al.*, 2000), suggesting that the pore formation may not be required for the activation of YxdJK. It may be essential to YxdJK activation that a peptide interacts directly and appropriately with the YxdK sensor in the membrane. In contrast, the LiaRS (YvqCE) TCS, which regulates *liaIHG* expression (Mascher *et al.*, 2004), was strongly activated by both peptides (LL-37 and PG-1) as well as by Triton X-100. Several other stress treatments, such as alkaline shock (Wiegert *et al.*, 2001), vancomycin (Cao *et al.*, 2002b) and secretion stress (H.-L. Hyyryläinen and others, unpublished results) also activate LiaRS (YvqCE). This further confirms that YxdJK senses a narrow range of signals or peptides, while LiaRS broadly senses various stress conditions. Interestingly, however, PLL did not activate LiaRS. These peptide ligands of known structure with differences in their specificity give an excellent future opportunity to study the structure–function relationships of these TCSs.

In addition to *yxdLM* and *liaIH* (*yvqIH*), some other genes of unknown function were also strongly induced by cationic antimicrobial peptides (DNA array). These included *yrhH*, which was induced by both LL-37 and PLL. The *yrhH* gene encodes a putative methyltransferase. *yhcG* was strongly upregulated by LL-37 (14·7-fold) and enhanced expression levels of several other genes of the *yhc* operon were also observed. *yhcG* encodes an ABC-transporter ATP-binding protein. The *yhcH* gene, which was induced 4·6-fold, also encodes a putative (second) ABC-transporter

ATP-binding protein, and the *yhcI* gene, which was induced 5·1-fold, encodes a putative ABC-transporter permease homologous with bacitracin permeases. The putative roles of these ABC transporters in the removal of LL-37 from cells should be studied in the future.

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