Int J Pept Res Ther (2009) 15:97–105
DOI 10.1007/s10989-009-9169-0

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Published online: 21 February 2009

Erratum to: Int J Pept Res Ther
DOI 10.1007/s10989-008-9157-9

Due to a production error, this article was previously published in the March 2009 issue of this journal (Vol. 15, No. 1).

The online version of the original article can be found under doi:10.1007/s10989-008-9157-9.

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Accepted: 26 September 2008

Abstract Using a novel methodology, we have investigated the transcriptional response of *Escherichia coli* to novispirin G10, an α-helical cationic antimicrobial peptide. We show that novispirin G10 induces an exceptionally coherent transcriptional response in *E. coli*, resulting in upregulation of genes involved in response to osmotic stress, acid shock, phage shock, and antimicrobial peptides, and down-regulation of the heat shock response genes, e.g., dnaJK, GroES, and GroEL. This transcriptional pattern indicates that novispirin G10 acts by compromising the bacterial membrane and possibly also by targeting the heat shock response. The impact of novispirin G10 on *E. coli* cells was monitored directly using the fluorescent LIVE/DEAD assay verifying that the peptide, indeed, targets bacterial membranes. Furthermore, in agreement with the observed heat shock transcriptional response, we show that overexpression of the heat shock transcription factor in *E. coli*, σ^{32}, leads to a significant decrease in sensitivity towards novispirin G10.

Keywords Novispirin G10 · Antimicrobial peptides · Mode-of-action · Transcriptional profiling · DNA microarray analysis

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Introduction

The rapid emergence of resistance to conventional antimicrobial agents is a growing problem in fighting bacterial and fungal infections. In contrast to conventional antibiotics, such as penicillin, development of resistance to antimicrobial peptides (AMP) is rare, and antimicrobial peptides therefore constitute an interesting alternative to marketed antibiotics (Coates et al. 2002; Zasloff 2002). Antimicrobial peptides are found in a variety of plants and animals, where they are part of a first line of defense against infections, making them ancient 'natural peptide antibiotics' (Ganz et al. 1985; Koczulla and Bals 2003; Nizet et al. 2001). Most antimicrobial peptides are cationic molecules, having a mode of action closely associated with their binding to negatively charged phospholipids that are exposed on the outer leaflet of bacterial membranes (Zasloff 2002). Several detailed models have been proposed to account for the rapid lysis of the target cells (Brogden 2005). However, there is mounting evidence indicating that antimicrobial peptides have multiple targets and that a subset of the peptides can traverse the bacterial membrane, exerting their bactericidal effects on intracellular targets as well as on the membrane (Brogden 2005; Hale and Hancock 2007; Hong et al. 2003; Otvos 2000; Patrzykat et al. 2002; Peschel and Sahl 2006; Wu et al. 1999; Yeaman and Yount 2003).

Here, we set out to investigate the transcriptional response and, thus, the mode of action of the AMP novispirin G10, which is a derivative of the N-terminal 18 amino acids of the sheep cathelicidin SMAP29 (Sawai et al. 2002). Novispirin G10 showed efficacy in several animal infection models against *S. aureus* and *P. aeruginosa* implicating its potential as an alternative antimicrobial therapeutic (Jacobsen et al. 2007; Song et al.

2005; Steinstraesser et al. 2002). Transcriptional profiling is a relatively new approach used to gain insight in the biological responses to various external physico-chemical stimuli. The method has been used successfully to fingerprint comprehendible cellular responses to sub-lethal exposure to traditional antibiotics (Ng et al. 2003). However, the method has been used with less success for antimicrobial peptides (AMPs) where the resulting profiles seem uncoordinated (Hong et al. 2003; Tomasinsig et al. 2004). This can be explained by several factors such as the speed of killing (within minutes) and the special feature of many AMPs where the concentration leading to inhibition (minimal inhibitory concentration—MIC) often is identical to the concentration leading to cell death (minimal bactericidal concentration—MBC).

Therefore, an alternative experimental setup where the bacterial cells were engineered to conditionally produce a periplasmic space targeted novispirin G10, thereby creating a local level of exposure to the AMP was employed. The rationale behind this approach is that as the AMP starts to build up and compromise the cellular integrity, the membrane potential and hence energy source for further AMP synthesis decreases. This results in a pseudo-steady state where the AMP-induced damage never reaches a level high enough to actually kill the cell. Through fine-tuning of the AMP synthesis, a controlled environment of maximum AMP-induced, sub-lethal stress is induced—the ideal background for studying the transcriptional response to the AMP. As a consequence of this novel strategy, the transcriptional response was exceptionally coherent and biologically relevant as shown directly by additional biochemical and genetic approaches.

Materials and Methods

Peptides

Peptides were produced by solid phase synthesis (K. J. Ross-Petersen, Denmark, www.ross.dk) and the corresponding sequences were novispirin G10: KNLRRIIRKG IHIIKKYG; IB-367: RGGLCYCRGRFCVCVGR-amide.

Plasmid Construction

Inducible novispirin G10 vector. A plasmid vector, pDRS5-novispirin G10 containing the novispirin G10 gene was constructed by annealing the DNA primers DR8F (5′-ccggccatggcgAAAAACCTGCGTCGCATTATCCGC AAAGGCATCCATATCATTAAAAAATATGGCtagatgg ctctagacggc-3′) and DRO (5′-GCCGTCTAGAGCCATC TA-3′) and filling out the DNA duplex using Taq Pwo polymerase (Roche). The resulting product was digested with *Nco*I/*Xba*I and ligated into the pBAD vector gIII A (Invitrogen). The construct was confirmed by sequence analyses and was transformed into *E. coli* TOP10 cells (Invitrogen).

Inducible RpoH-Vector

The rpoH-gene was PCR amplified from *E. coli* TOP10 using the DNA primers rpoH-forw1 (5′-taacaggagga attaaccatgatgactgacaaaatgcaaagtttagcttt-3′) and rpoH-rev (5′-tttgttctagattacgcttcaatggcagcgcgcaatttttttcatcgcg-3′). A DNA fragment of approximately 880 bp was purified and used as template in another PCR reaction using the DNA primers rpoH-forw2 (3′-tagcggatcctacctgacgctttttatcgcaact ctctactgtttctccatacccgttttttgggctaacaggaggaattaaccatgatgact gac-5′) and rpoH-rev. The amplified fragment was purified and restricted with *Nco*I and *Xba*I and cloned into the plasmid pBAD gIII A. The correct sequence was verified by DNA sequencing.

Preparation of Bacterial Cultures for Microarray Analyses

E. coli TOP10 cells (Invitrogen) transformed either with the novispirin G10 expressing construct (pDRS5-novispirin G10) or the control vector were grown to exponential phase in RM media (see Invitrogen catalog no. V450-01) supplemented with 100 mg/l ampicillin and 0.1% arabinose (Ferro-Pfanstiehl A-121) and subsequently transferred to centrifugation tubes containing 10 ml pre-frozen milliQ water. Cells were immediately harvested by centrifugation at 4,200×*g* for 15 min at 4°C. The supernatant was decanted and the pelleted cells were frozen at −80°C until RNA extraction was performed.

RNA Isolation and Labelling

Total RNA was isolated from the two *E. coli* cultures (expressing either novispirin G10 or a control peptide) by use of the High Pure RNA Isolation kit (Roche, cat #1828665) according to the manufacturer's instructions. Residual DNA was removed on-column with RNase-free DNase. Labelled samples were prepared from 30 µg of total bacterial RNA. Fluorescent first strand cDNA was prepared by random primed reverse transcription (Superscript II; Life Technologies) by use of random hexamers for cDNA synthesis. A total of 2 µl (20 µg) of random primer was mixed with 15 µl (30 µg) of total RNA, incubated for 5 min at 70°C, and cooled on ice. To each sample was added 5× first strand buffer (6 µl), 0.1 M DTT (3 µl), dNTP mix (5 mM dATP, dGTP and dTTP and 2 mM dCTP) (1 µl), RNaseOut (1 µl), Superscript II reverse transcriptase (2 µl) and Cy-dCTP (3 µl). Reverse

transcription was carried out at 42°C for 1 h. RNA was removed by addition of 10 µl NaOH (1 M) and incubation at 65°C for 10 min. The samples were neutralised by addition of 10 µl HCl (1 M). Cy3 and Cy5 cDNA samples were mixed and immediately purified with a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences).

Hybridizations, Scanning and Analysis

Slides were pre-hybridised at 42°C for 45 min in a solution containing 4× SSC + 0.5% SDS + 1% BSA and dried by centrifugation. Hybridizations were in a volume of 25 µl under a supported cover slip at 42°C for 16–18 h at high humidity. The hybridisation mixture was labelled cDNA in a formamide-based hybridisation buffer (MWG-supplied with the pan-arrays). Cover slip and supports were gently removed (2× SSC in a wash tray) and arrays were washed by immersion into 2× SSC + 0.1% SDS for 5 min, 1× SSC + 0.1% SDS for 5 min, 0.5× SSC for 5 min and 1× millipore water for 10 s and dried by centrifugation. Slides were scanned on a GMS 418 scanner to detect Cy3 and Cy5 fluorescence. Data analysis was carried out using ImaGene 5.0 and GeneSight 3.5 from BioDiscovery (www.biodiscovery.com). Two biological replicate experiments with four replicate hybridisations in each experiment were carried out for generation of data.

Pan *E. coli* K12 Arrays from MWG Biotech were used in this study (lot No.:020206). The 4239 *E. coli* K12 specific oligonucleotide probes present on this glass-based DNA chip represent the complete *E. coli* (K12) genome

BacLight Live/Dead Membrane Permeabilization Assay

E. coli MG1655 cells were grown for at least six mass doublings in Mueller-Hinton Broth (MHB) at 37°C until an OD_{600} of approximately 0.5. Aliquots of 200 µl of cells were transferred to a V96 microwell plate (NUNC) and supplemented with a mixture of SYTO 9 and propidium iodide from the BacLight Bacterial Viability Kit L7007 as described by the manufacturer (Molecular probes). Green fluorescence was measured for 5 min using FLUOstar OPTIMA equipment (BMG LABTECH) with an excitation and emission wavelength of 485 and 510 nm, respectively. Subsequently, various antibacterial compounds were added to the individual wells as indicated at concentrations of 2–4× the MIC. Green fluorescence readings were recorded over the next 55 min at 30 s intervals.

Radial Diffusion Assay

The assay used in this study was based on the protocols described previously (Lehrer et al. 1991) with several modifications. *E. coli* TOP10 (Invitrogen) cultures containing either the control vector or the sigma-32 overexpressing construct were grown overnight at 37°C in RM media supplemented with 100 mg/l ampicillin. Next day, duplicates of each culture were grown to exponential phase in the absence or in the presence of 0.1% arabinose. Subsequently, 3×10^6 CFU from each culture were spread on the surface of a thin layer of a 1% agarose gel (SIGMA A-6013) containing Mueller-Hinton broth (MHB, Becton Dickinson 212322). A regularly spaced array of wells was made in the MHB agarose gel. Next, 10 µl of six serially twofold diluted samples of each peptide ranging in concentration from 200 to 6.25 µg/ml in 0.01% acetic acid and 0.1% bovine serum albumin (Roche 775860) were added to the wells. The plates were incubated overnight at 37°C to allow bacterial growth. Finally, the resulting clearing zones were measured by a circle array program. Triplicates of each sample were run in the assay. The experiment was performed four independent times for novispirin G10 and twice for IB-367.

Results and Discussion

Based on a confidence level of 90%, microarray analysis showed that 153 genes were upregulated and 92 genes were downregulated relative to a control strain that expressed a peptide without antimicrobial activity. The majority of the annotated, upregulated genes could be ascribed to a small number of physiological responses (Table 1), comprising responses to membrane dysfunction—i.e., upregulation of genes involved in acid shock, osmotic shock and the respiratory system—and functional groups related to phage shock, AMP resistance, the magnesium stimulon, nitrogen assimilation, and the carbon metabolism.

In line with the expectation of a membrane destabilizing action of an antimicrobial peptide, the transcriptional response elicited by novispirin G10 shows clear signs of compromised membrane characteristics, as indicated by upregulation of genes involved in osmotic shock response, acid resistance and the electron transport system (Yeaman and Yount 2003), (Table 1). The upregulated genes responsible for counteracting the osmotic shock include a proline/betaine transporter, *proW*, a putative osmoprotectant system, *yehZYXW* (Checroun and Gutierrez 2004), and the trehalose biosynthetic enzymes, encoded by the *otsAB* operon. At high osmolality, betaine and proline act as osmoprotectants when available in the medium, and if not available, the intracellular level of the neutral solute,

Table 1 Up- and down-regulated genes in response to novispirin G10

Gene	b-number	Description	Functional group	Fold	Confidence level (%)
dksA	b0145	dnaK suppressor protein	Acid shock	4.1	95
ybaS	b0485	Putative glutaminase	Acid shock	8.8	99
nmpC	b0553	Outer membrane protein, negatively regulated during growth at low pH	Acid shock	−6.6	99
dps	b0812	DNA binding protein; global regulator, starvation conditions	Acid shock	4.4	95
ycaC	b0897	orf, hypothetical protein	Acid shock	7.4	99
gadC	b1492	Putative glutamate:GABA antiporter (*xasA*)	Acid shock	24.9	99
asr	b1597	Periplasmic acid shock protein	Acid shock	19.2	99
ydiZ	b1724	ORF, hypothetical protein	Acid shock	9.1	99
yeaQ	b1795	Putative transglycosylase-associated membrane protein, acid inducible	Acid shock	6.9	99
yebV	b1836	ORF, hypothetical protein	Acid shock	6.9	99
gabT	b2662	GABA aminotransferase	Acid shock	6.5	99
gabP	b2663	GABA permease	Acid shock	8.2	99
slp	b3506	Outer membrane protein induced after carbon starvation	Acid shock	3.6	90
yhif	b3507	ORF, hypothetical protein	Acid shock	2.9	80
hdeB	b3509	Periplasmic protein involved in acid resistance	Acid shock	7.9	99
hdeA	b3510	Periplasmic protein involved in acid resistance	Acid shock	7.7	99
hdeD	b3511	Integral membrane protein, unknown function, acid inducible	Acid shock	4.3	95
gadE	b3512	Activates glutamate-dependent acid resistance (*yhiE*)	Acid shock	3.9	90
gadW	b3515	Transcriptional regulator, AraC-type (*yhiW*)	Acid shock	3.0	80
yiaG	b3555	Putative transcription factor, acid inducible	Acid shock	10.1	99
bolA	b0435	Morphogene, induced by osmotic shock	Osmotic shock	13	99
dacC	b0839	D-alanyl-D-alanine carboxypeptidase; regulated by *bolA*	Osmotic shock	3.6	90
osmC	b1482	Osmotically induced protein	Osmotic shock	7.2	99
katE	b1732	Catalase, hydroperoxidase HPII(III)	Osmotic shock	9.9	99
osmE	b1739	Osmotically inducible, activator of ntrL	Osmotic shock	11.7	99
otsA	b1896	Trehalose-6-phosphate synthase	Osmotic shock	5.2	95
otsB	b1897	Trehalose-6-phosphate phosphatase	Osmotic shock	5.6	99
yehX	b2129	Part of putative osmoprotectant system, yehZYXW	Osmotic shock	5.6	99
yehY	b2130	Part of putative osmoprotectant system, yehZYXW	Osmotic shock	4.2	95
yehZ	b2131	Part of putative osmoprotectant system, yehZYXW	Osmotic shock	7.1	99
proW	b2678	High affinity transport system for glycine, betaine and proline	Osmotic shock	7.9	99
osmY	b4376	Hyperosmotically inducible periplasmic protein	Osmotic shock	6.4	99
appC	b0978	Cytochrome bd terminal oxidase, subunit I	Redox	3.5	90
appB	b0979	Cytochrome bd terminal oxidase, subunit II	Redox	47.2	99
ndh	b1109	Transfer of electrons from NADH to the respiratory chain	Redox	3.1	80
aer	b3072	Aerotaxis sensor receptor	Redox	14.3	99
pspA	b1304	Phage shock protein	Phage shock	6.7	99
pspB	b1305	Phage shock protein	Phage shock	6.9	99
pspC	b1306	Phage shock protein	Phage shock	15.8	99
pspD	b1307	Phage shock protein	Phage shock	13.5	99
pspE	b1308	Phage shock protein	Phage shock	9.4	99
dnaK	b0014	Heat shock protein, chaperone	Heat shock	−2.8	80
dnaJ	b0015	Heat shock protein, chaperone	Heat shock	−3.0	90
lon	b0439	Heat shock protein, protease	Heat shock	−2.9	80
clpB	b2592	Heat shock protein, chaperone	Heat shock	−3.6	95
grpE	b2614	Heat shock protein, stimulates DnaK ATPase	Heat shock	−3.0	90
ibpB	b3686	Heat shock protein, chaperone	Heat shock	−6.5	99

Table 1 continued

Gene	b-number	Description	Functional group	Fold	Confidence level (%)
hslU	b3931	Heat shock protein, protease	Heat shock	−3.2	90
hslV	b3932	Heat shock protein	Heat shock	−3.0	90
mopB	b4142	Heat shock protein, GroES, chaperone	Heat shock	−9.1	99
mopA	b4143	Heat shock protein, GroEL, chaperone	Heat shock	−3.6	95
sfmC	b0531	Periplasmic pilus chaperone	Protein folding	11.4	99
crcA	b0622	phoP/phoQ-dependent enzyme, lipid A modifying enzyme	AMP resistance	11.7	99
ais	b2252	Homolog to polymyxin resistance gene *pmrG* in *S. typhimurium*	AMP resistance	14.1	99
b2253	b2253	Putative aminotransferase; modification of lipid A with aminoarabinose	AMP resistance	8.4	99
b2254	b2254	Putative glycosyl transferase; LPS biosynthesis (*pmrF* homolog)	AMP resistance	5.5	99
b2256	b2256	Homolog to polymyxin resistance gene in *S. typhimurium*	AMP resistance	4.0	90
hemL	b0154	Glutamate-1-semialdehyde aminotransferase	Mg^{2+}-stimulon	8.3	99
nagA	b0677	*N*-acetylglucosamine-6-phosphate deacetylase	Mg^{2+}-stimulon	3.7	90
nagB	b0678	Glucosamine-6-phosphate deaminase	Mg^{2+}-stimulon (?)	8.8	99
phoQ	b1129	Part of two-component regulatory system with *phoP*	Mg^{2+}-stimulon	3.7	90
phoP	b1130	Sensor in two-component regulatory system with *phoQ*	Mg^{2+}-stimulon	2.8	80
rstB	b1609	Putative sensor kinase	Mg^{2+}-stimulon	3.6	90
mgrB	b1826	Hypothetical protein; induced by low magnesium	Mg^{2+}-stimulon	4.6	95
mgtA	b4242	Magnesium transport, PhoP/PhoQ-dependent	Mg^{2+}-stimulon	7.5	99
narX	b1222	Nitrate/nitrate sensor, histidine protein kinase	Nitrogen assimilation	−4.0	95
narY	b1467	Cryptic nitrate reductase 2, beta subunit	Nitrogen assimilation	11.0	99
narZ	b1468	Crytic nitrate reductase 2, alpha subunit	Nitrogen assimilation	4.9	95
narU	b1469	Nitrite extrusion protein 2	Nitrogen assimilation	15.1	99
nirB	b3365	Nitrite reductase, NAD(P)H, large subunit	Nitrogen assimilation	−2.9	80
nirD	b3366	Nitrite reductase (NAD(P)H) subunit	Nitrogen assimilation	−5.7	99
nirC	b3367	Potential nitrite transporter	Nitrogen assimilation	−3.0	90
nrfA	b4070	Component of nitrite reductase complex	Nitrogen assimilation	−12.7	99
sfsA	b0146	Probable regulator for maltose metabolism	Carbon metabolism	6.4	99
sfcA	b1479	Putative NAD-linked malic enzyme	Carbon metabolism	5.8	99
amyA	b1927	Cytoplasmic alpha-amylase	Carbon metabolism	5.8	99
b2097	b2097	Class I Fructose 1,6 bisphosphate aldolase	Carbon metabolism	8.1	99
glk	b2388	Glucokinase	Carbon metabolism	3.4	80
talA	b2464	Transaldolase A	Carbon metabolism	11.7	99
tktB	b2465	Transketolase 2 isoenzyme	Carbon metabolism	26.5	99

trehalose, is increased through biosynthesis (Yancey 2005). Along with these solute accumulating processes, osmotic stress is evidenced by the upregulation of the osmotically inducible genes of *osmC*, *osmE* and *osmY*.

Several genes responsive to low pH were found among the most upregulated genes. The most upregulated acid resistance gene is *gadC*, which is part of the most effective of three distinct acid resistance systems present in *E. coli*, the GAD-system (Castanie-Cornet et al. 1999; Tucker et al. 2002). The GAD-system is glutamate dependent, comprising a glutamate decarboxylase, encoded by *gadB* or *gadA*, and a membrane-associated γ-aminobutyric acid (GABA): glutamate antiporter, encoded by *gadC*. According to this model, accumulation of GABA—in the case of insufficient extracellular glutamate for the GABA:glutamate antiporter system—is prevented by the export of GABA by the GABA permease encoded by *gabP*. Alternatively, GABA may be directed towards degradation in the central metabolism by the action of GABA aminotransferase, the first of two steps converting GABA to succinate, a tricarboxylic acid cycle intermediate. In the present case, the GAD-system is accompanied by an auxiliary enzyme, encoded by *ybaS* that may provide glutamate from glutamine through the action of a glutaminase (Fig. 1). Comparison of the upregulated genes in Table 1 with the results from whole-genome expression

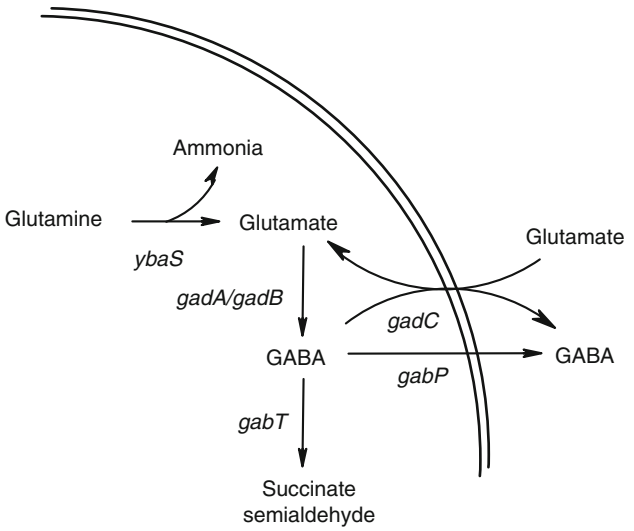

Fig. 1 Model for genes involved in acid resistance in response to novispirin G10

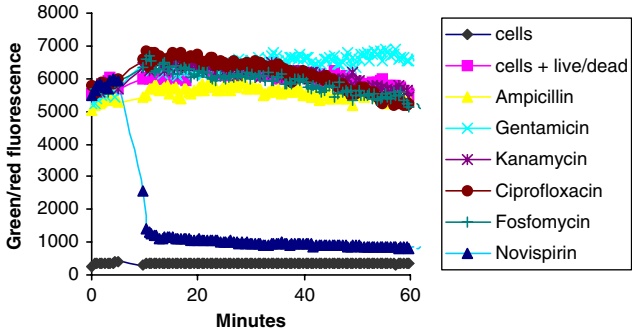

Fig. 2 Novispirin G10 permeabilizes the membranes of *E. coli* strain MG1655 as demonstrated using LIVE/DEAD staining. The effect of novispirin was compared with the reference antibiotics indicated. Values on Y-axis depict the green/red fluorescence ratio. All compounds were used at a concentration of 2–4× the MIC. Bacterial cells were grown and assayed in MHB medium

profiling of acid inducible genes in *E. coli* shows that out of 31 acid-induced genes, 18 were found to be upregulated in this study (Tucker et al. 2002).

Impaired ability to maintain gradients over the membrane also affects the redox level, explaining the 14-fold upregulation of *aer*, an aerotaxis sensor receptor believed to sense redox changes in the electron transport system (Taylor and Zhulin 1998). Other upregulated genes in the respiratory system are *appBC*, encoding a cytochrome bd terminal oxidase, and *ndh*, encoding an NADH dehydrogenase.

Since the mode of action of cationic antimicrobial peptides is dependent on their affinity for negatively charged groups on the cell membrane, lowering the electrostatic interactions between cationic antimicrobial peptides and the membrane components is a way for the bacteria to circumvent the action of antimicrobial peptides (Gunn et al. 1998; Yeaman and Yount 2003). Thus, in *Salmonella* spp., several genes involved in resistance to polymyxin, a cationic antimicrobial peptide, have been reported to code for enzymes that modify lipid A—a main constituent of the outer leaflet of the outer membrane—in a way that decreases the number of negative charges (Gunn et al. 1998). In this study, five genes were found to be involved in resistance to antimicrobial peptides. Four of these genes, *ais* (*b2252*), *b2253*, *b2254* and *b2256*, map to a region of the *E. coli* genome that is homologous to the polymyxin resistance operon of *S. typhimurium* (Gunn et al. 1998). In addition to these genes, *crcA*—another lipid A modifying gene regulated by PhoP–PhoQ (Bishop et al. 2000)—was found to be upregulated.

To directly examine the effect of novispirin G10 on bacterial membranes we subsequently used the LIVE/DEAD fluorescent assay (Materials and Methods). Briefly, the assay employs a mixture of two nucleic acid stains, the green-fluorescent SYTO 9 and the red-fluorescent propidium iodide stain. When these dyes are used together, cells with intact membranes show green fluorescence, while in cells with damaged membranes, propidium iodide enters the cells and quenches the green fluorescence. Treating *E. coli* cells with Novispirin G10 resulted in an immediate decrease in the green fluorescence (Fig. 2). On the other hand *E. coli* cells treated with the cell wall active antibiotics (phosphomycin and ampicillin) or compounds that target intracellular processes such as protein (gentamicin and kanamycin) or DNA (ciprofloxacin) synthesis remained intact over the entire course of the experiment. Thus, novispirin G10 clearly compromises membrane integrity of bacterial cells verifying the results obtained by the transcriptional profiling.

The phage shock genes encoded by the *pspABCDE* operon were all highly upregulated in response to novispirin G10. The *psp* operon is sigma-54 dependent and it is known to be induced by various types of stress, including hyperosmotic shock, uncoupling of the oxidative phosphorylation and nutritional downshifts (Reitzer and Schneider 2001). Stress caused by heat shock also leads to upregulation of the *psp*-operon, an upregulation that is even further enhanced in cells with impaired ability to mount a heat shock response (Brissette et al. 1990). The finding that genes encoding central heat shock proteins, including DnaJK, GroEL, GroES, and IbpB, are downregulated (see Table 1) suggests that novispirin G10 producing cells may not elicit a normal heat shock response, thereby enhancing the upregulation of the *psp*-operon. Also suggestive of a reduced level of heat shock proteins, the *dnaK* suppressor gene, *dksA*, was found to be upregulated. The apparent lack of a normal heat shock response may seriously affect the viability of cells growing at 37°C, and the downregulation

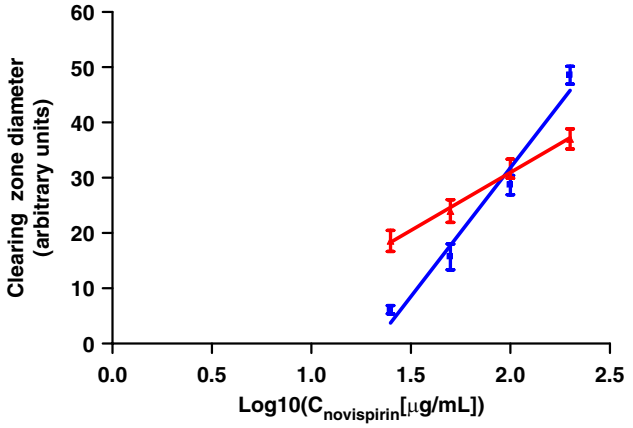

Fig. 3 Clearing zone diameter from a radial diffusion assay plotted against the logarithm to the novispirin concentration (μg/ml). The blue squares represent experiments in which the cells were induced by arabinose prior to the radial diffusion assay, and the red triangles represent experiments in which there was no arabinose dependent induction of σ^{32}. Extrapolation to the intercept with the x-axis indicates the value of the (logarithm to the) minimal effective concentration (MEC)

of the heat shock genes may therefore be part of the mechanism by which novispirin G10 kills *E. coli*.

To investigate this, a strain conditionally overexpressing the heat shock transcription factor, sigma-32, was constructed. Growing the strain under inducing conditions before plating the strain onto Mueller-Hinton Broth (MHB) agarose plates, increased the minimal effectiv concentration (MEC) of novispirin in a radial diffusion assay from 3.9 (±2.9) μg/ml in the non-induced case to 20.9 (±1.8) μg/ml in the case of induction (Fig. 3), indicating that the upregulation of the heat shock genes makes the strain less susceptible to novispirin G10. The increased MEC supports the notion that short cationic antimicrobial peptides do not solely exert their bactericidal effect through destabilization of the membrane (Patrzykat et al. 2002). Overexpression of sigma-32 reduced the growth rate, and to separate the effect of growth rate reduction from the effect of sigma-32 overexpression, a control experiment was performed with IB-367, an antimicrobial peptide belonging to a different class of cationic antimicrobial peptides. In this experiment, sigma-32-overexpression did not increase the MEC value, indicating that sigma-32 overexpression does not affect the resistance to antimicrobial peptides in general.

We have thus demonstrated that the transcriptional response of *E. coli* to novispirin G10 is a highly coordinated response aimed at fending off the AMP while at the same time compensating for a damaged membrane. Also the heat shock response was shown to be affected by novispirin G10, and increasing the level of the heat shock transcription factor more than doubled the minimal effective concentration (MEC) of novispirin G10. The large number of genes involved in the response to a destabilised membrane shows that the primary target of novispirin G10 is the membrane. However, the action of novispirin G10 may also be potentiated by having the heat shock response as a secondary target.

Acknowledgments We thank I. Ellingsgaard, A. L. Hansen and J. D. Larsen for expert technical assistance. *Funding*: The work was funded by Novozymes A/S. *Transparency declarations*: The authors are employees of Novozymes A/S. The authors declare that they have no other competing financial interest.

References

Bishop RE, Gibbons HS, Guina T, Trent MS, Miller SI, Raetz CR (2000) Transfer of palmitate from phospholipids to lipid A in outer membranes of gram-negative bacteria. EMBO J 19(19):5071–5080

Brissette JL, Russel M, Weiner L, Model P (1990) Phage shock protein a stress protein of *Escherichia coli*. Proc Natl Acad Sci USA 87(3):862–866

Brogden KA (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 3(3):238–250

Castanie-Cornet MP, Penfound TA, Smith D, Elliott JF, Foster JW (1999) Control of acid resistance in *Escherichia coli*. J Bacteriol 181(11):3525–3535

Checroun C, Gutierrez C (2004) Sigma(s)-dependent regulation of yehZYXW which encodes a putative osmoprotectant ABC transporter of *Escherichia coli*. FEMS Microbiol Lett 236(2):221–226

Coates A, Hu Y, Bax R, Page C (2002) The future challenges facing the development of new antimicrobial drugs. Nat Rev Drug Discov 1(11):895–910

Ganz T, Selsted ME, Szklarek D, Harwig SS, Daher K, Bainton DF, Lehrer RI (1985) Defensins natural peptide antibiotics of human neutrophils. J Clin Invest 76(4):1427–1435

Gunn JS, Lim KB, Krueger J, Kim K, Guo L, Hackett M, Miller SI (1998) PmrA–PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. Mol Microbiol 27(6):1171–1182

Hale JD, Hancock RE (2007) Alternative mechanisms of action of cationic antimicrobial peptides on bacteria. Expert Rev Anti Infect Ther 5(6):951–959

Hong RW, Shchepetov M, Weiser JN, Axelsen PH (2003) Transcriptional profile of the *Escherichia coli* response to the antimicrobial insect peptide cecropin A. Antimicrob Agents Chemother 47(1):1–6

Jacobsen F, Mohammadi-Tabrisi A, Hirsch T, Mittler D, Mygind PH, Sonksen CP, Raventos D, Kristensen HH, Gatermann S, Lehnhardt M, Daigeler A, Steinau HU, Steinstraesser L (2007) Antimicrobial activity of the recombinant designer host defence peptide P-novispirin G10 in infected full-thickness wounds of porcine skin. J Antimicrob Chemother 59(3):493–498

Koczulla AR, Bals R (2003) Antimicrobial peptides: current status and therapeutic potential. Drugs 63(4):389–406

Lehrer RI, Rosenman M, Harwig SS, Jackson R, Eisenhauer P (1991) Ultrasensitive assays for endogenous antimicrobial polypeptides. J Immunol Methods 137(2):167–173

Ng WL, Kazmierczak KM, Robertson GT, Gilmour R, Winkler ME (2003) Transcriptional regulation and signature patterns revealed by microarray analyses of *Streptococcus pneumoniae* R6

challenged with sublethal concentrations of translation inhibitors. J Bacteriol 185(1):359–370

Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudisill J, Dorschner RA, Pestonjamasp V, Piraino J, Huttner K, Gallo RL (2001) Innate antimicrobial peptide protects the skin from invasive bacterial infection. Nature 414(6862):454–457

Otvos LOI Jr, I O, Rogers ME, Consolvo PJ, Condie BA, Lovas S, Bulet P, Blaszczyk-Thurin M (2000) Interaction between heat shock proteins and antimicrobial peptides. Biochemistry 39:14150–14159 46 pp

Patrzykat A, Friedrich CL, Zhang L, Mendoza V, Hancock RE (2002) Sublethal concentrations of pleurocidin-derived antimicrobial peptides inhibit macromolecular synthesis in *Escherichia coli*. Antimicrob Agents Chemother 46(3):605–614

Peschel A, Sahl HG (2006) The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nat Rev Microbiol 4(7):529–536

Reitzer L, Schneider BL (2001) Metabolic context and possible physiological themes of sigma(54)-dependent genes in *Escherichia coli*. Microbiol Mol Biol Rev 65(3):422–444 table

Sawai MV, Waring AJ, Kearney WR, McCray PB Jr, Forsyth WR, Lehrer RI, Tack BF (2002) Impact of single-residue mutations on the structure and function of ovispirin/novispirin antimicrobial peptides. Protein Eng 15(3):225–232

Song Z, Wu H, Mygind P, Raventos D, Sonksen C, Kristensen HH, Hoiby N (2005) Effects of intratracheal administration of novispirin G10 on a rat model of mucoid *Pseudomonas aeruginosa* lung infection. Antimicrob Agents Chemother 49(4):3868–3874

Steinstraesser L, Tack BF, Waring AJ, Hong T, Boo LM, Fan MH, Remick DI, Su GL, Lehrer RI, Wang SC (2002) Activity of novispirin G10 against *Pseudomonas aeruginosa* in vitro and in infected burns. Antimicrob Agents Chemother 46(4):1837–1844

Taylor BL, Zhulin IB (1998) In search of higher energy: metabolism-dependent behaviour in bacteria. Mol Microbiol 28(4):683–690

Tomasinsig L, Scocchi M, Mettulio R, Zanetti M (2004) Genome-wide transcriptional profiling of the *Escherichia coli* response to a proline-rich antimicrobial peptide. Antimicrob Agents Chemother 48(9):3260–3267

Tucker DL, Tucker N, Conway T (2002) Gene expression profiling of the pH response in *Escherichia coli*. J Bacteriol 184(23):6551–6558

Wu M, Maier E, Benz R, Hancock RE (1999) Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli*. Biochemistry 38(22):7235–7242

Yancey PH (2005) Organic osmolytes as compatible metabolic and counteracting cytoprotectants in high osmolarity and other stresses. J Exp Biol 208(15):2819–2830

Yeaman MR, Yount NY (2003) Mechanisms of antimicrobial peptide action and resistance. Pharmacol Rev 55(1):27–55

Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature 415(6870):389–395