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Cationic peptide antimicrobials induce selective transcription of *micF* and *osmY* in *Escherichia coli*

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Abstract

Cationic antimicrobial peptides, such as polymyxin and cecropin, activated transcription of *osmY* and *micF* in growing *Escherichia coli* independently of each other. The *micF* response required the presence of a functional *rob* gene. It is intriguing that in this and other assays an identical response profile was also seen with hyperosmotic salt or sucrose gradient, two of the most commonly used traditional food preservatives. The *osmY* and *micF* transcription was not induced by hypoosmotic gradient, ionophoric peptides, uncouplers, or with other classes of membrane perturbing agents. The antibacterial peptides did not promote transcription of genes that respond to macromolecular or oxidative damage, fatty acid biosynthesis, heat shock, or depletion of proton or ion gradients. These and other results show that the antibacterial cationic peptides induce stasis in the early growth phase, and the transcriptional efficacy of antibacterial peptides correlates with their minimum inhibitory concentration, and also with their ability to mediate direct exchange of phospholipids between vesicles. The significance of these results is developed as the hypothesis that the cationic peptide antimicrobials stress growth of Gram-negative organisms by making contacts between the two phospholipid interfaces in the periplasmic space and prevent the hyperosmotic wrinkling of the cytoplasmic membrane. Broader significance of these results, and of the hypothesis that the peptide mediated contacts between the periplasmic phospholipid interfaces are the primary triggers, is discussed in relation to antibacterial resistance.

Keywords: Antibacterial peptides; Antibiotic resistance; Bacterial stasis; Plasmolysis; Osmotic stress

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; EMS, ethyl methanesulfonate; $FC_{1\%}$, the fluorescence increase induced by 1 mol% peptide in the PyPG exchange assay; LPS, lipopolysaccharide; MIC, minimum growth inhibitory concentration in 60 min; NP, polymyxin B (2–10)-nonapeptide; OM, outer membrane Gram-negative organism; POPG, 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphoglycerol; PxB, polymyxin B; PyPG, 1-palmitoyl-2-pyrenedecanoyl-phosphatidylglycerol; TLRC, threshold luminescence response concentration

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1. Introduction

Ubiquitous antimicrobial peptides [1–5] are a part of the innate immune system [6] of virtually all organisms. Some of the better characterized examples include polymyxins of Gram-positive *Polymyxa* spp. [7], magainins of frog skin [6,8–10], cecropins of insect larvae [11,12], thionins of plants [13], and defensins from humans and other animals [2,14]. Evolutionary success of antimicrobial strategies with target selectivity suggests solution to the problem of antibiotic resistance that could provide insights into the management of infection.

It is generally believed that the bacterial membrane is the primary target of antimicrobial cationic peptides [2,7]. The fact that these antimicrobials do not cause significant damage to organisms that produce them, suggests specific mechanisms of action, rather than a lytic mechanism that causes leakage of cytoplasmic content. We have developed strategies and protocols to determine the metabolic stress induced in growing *Escherichia coli* by antimicrobials [15,16]. The conceptual basis for this assay lies in the fact that viable organisms often respond and adapt to sub-lethal environmental adversities by increased expression of stress proteins to restore homeostasis [17,18]. The transcription of a specific stress promoter is obligatorily coupled to a bacterial luminescence reporter *luxCDABE* operon on a plasmid introduced in *E. coli*. Such fusion strains (Table 1) produce luminescence at sub-lethal level of the stress [19–27]). Specific stress response is measured with cells in the growth phase treated with concentrations of the antimicrobial agent below its growth inhibitory concentrations. Under these conditions the growth-dependent metabolic processes are stressed but are not turned off, therefore cells respond and achieve homeostasis. By design, this method provides a measure of the physiological change that requires onset of transcription with the response time of several minutes at the sub-lethal doses. Extension of the stress–response results to establish the antimicrobial basis derives from the assumption that continued excessive stress leads to bacteriostasis, and ultimately to cell death.

In this paper we characterize the nature of the stress induced by several cationic peptides on *E. coli*. The promoter-coupled luminescence technique showed that the stress induced by cationic peptides like PxB selectively enhanced the expression of *micF* encoding an antisense RNA that inhibits expression of the OmpF porin, and *osmY* encoding an osmotically-induced periplasmic protein. Expression initiated at these two promoters was also induced by hyperosmolar NaCl or sucrose, two of the most commonly used food preservatives. Although transcription of *micF* and *osmY* is influenced by similar factors, the effect is independent of each other. The significance of the primary interaction with phospholipid targets is discussed in relation to antibiotic resistance.

2. Materials and methods

2.1. Reagents and growth media

PxB, bacitracin, gramicidin A, gentamicin, valinomycin, and agarose were from Sigma. Magainins, cecropins and mastoparans were from Bachem. Colistin was from Waku Chemical Co. PxB-agarose and NP were from Boehringer. CCCP was provided by Dr. P. Heytler (DuPont Co.). The purity of the peptides was confirmed by analytical HPLC. Sources of phospholipid and protocols to monitor phospholipid exchange is described in detail elsewhere [40,41]. The M9 medium contained (per liter) 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl; after autoclaving 1 ml of 1 M $MgSO_4$, 0.1 ml of 1 M $CaCl_2$, 10 ml of 20% glucose, and 1 mg thiamine were added. LB medium (Sigma) was used as described [15,16].

2.2. Fusion strains, growth conditions and luminescence response

The panel of stress responsive *lux* fusion strains used in this study is listed in Table 1. Plasmid pMicFLux1 containing a fusion of the *micF* promoter region to the *Vibrio fischeri luxCDABE* operon in the parental plasmid pUCD615 [28] was made by PCR amplification according to a previously described method [24] using the primers: 5′-ACTTAAGGATCCCCCCAAAAATGCAGAATA-3′ and 5′-AGCAGCGAATTCGGGCATCCGGTTGAAATAG-3′. The amplified product contains 244 base

pairs upstream of the start site of the *micF* RNA and the entire transcribed region.

A series of *E. coli* strains that are isogenic with the exception of chromosomal mutations in the genes encoding certain regulatory proteins were used to test their effects on *micF* expression. *E. coli* strains DPD2191, DPD2192, DPD2193 and DPD2194 were made by transformation using plasmid pMicF-Lux1 into *E. coli* strains GC4468 (*F-Δlac4169 rpsL*) [30], N7840 (*F-Δlac4169 rpsL Δ(mar sad)1738*) [31], BW829 (*F-Δlac4169 rpsL Δsox-8::cat*) [32], and RA4468 (*F-Δlac4169 rpsL rob::kan*) [30], respectively. Strain DPD2220 was constructed by transformation of pDEW221 with the *osmY′-luxCDABE* fusion into strain RA4468 with the chromosomal *rob*− mutation.

The transcriptional responses were assayed by previously established protocols [16]. Briefly, turbidity and luminescence measurements were carried out at 30°C with shaking at 200 rpm in LB medium containing 50 μg/ml kanamycin monosulfate or ampicillin to maintain the plasmid. The OD (Spectronix 2000, Bausch and Lomb) at 600 nm of the culture (OD_{600}) was measured after 5- to 15-fold dilution in the medium at the indicated intervals. When OD_{600} reached 0.2, 5 ml culture broth was transferred to a 25 ml flask, followed by the addition of appropriate concentration of a prefiltered (0.2 μm filter) inducer (Table 2), or peptide, or suitable controls. Controls with NP were based on the observation that NP interacts with the bacterial LPS layer but it is a very weak antibacterial. Luminescence of the culture broth was monitored without dilution by transferring a 0.2 ml aliquot to 1.5 ml polyethylene tubes (Turner Design) on a Model 20e Turner Design luminometer pre-set at a constant sensitivity. For comparison purposes the signal intensities in arbitrary units are expressed as L/L_C-1, where L is the observed luminescence under a given set of conditions for the stress variable, and L_C is the control luminescence without the stress. The error bars are not shown; however, replicate assays were routinely carried out. Typically the scatter was less than 10% in the absolute reading; however, the luminescence changes significantly with the growth conditions. Independent controls were also carried out to show that the cells are viable after the luminescence measurements.

Comparable growth and assay conditions were used to monitor the effect of washed PxB-agarose [7] suspended in the autoclaved LB medium and shaken for 30 min. Similarly, cultures of *E. coli* or several *Pseudomonas* species in the early log phase were transferred to fresh medium containing PxB at its MIC, and then at successively higher concentrations. Analysis of the growth medium showed that $>80\%$ of the peptide was still intact and active. Fatty acid analysis to identify bacterial strains was carried out as described [37]. Standard protocols were used for UV and chemical (EMS) mutagenesis [38].

2.3. Effects of antimicrobial peptides on the biophysical properties and phospholipid exchange

Strategies, protocols and controls for the phospholipid exchange and other biophysical effects of PxB [39–41] have been established. Such protocols distinguish the peptide-mediated leakage of the aqueous content, (hemi-)fusion of vesicles, solubilization of the bilayer, transbilayer movement, and the exchange of phospholipid between the outer monolayers of vesicles kept in contact through a peptide or protein. Pyrene-labeled phospholipid dequenching assay was used to quantify the peptide contact mediated phospholipid exchange. Typically, the fluorescence increase at 396 nm (excitation 346 nm) was monitored as a function of time after the addition of a known mole fraction of the peptide to a mixture of pyPG/POPC (70:30) covesicles with a 125-fold excess of unlabeled phospholipid covesicles of DMPG/POPC (70:30). The total concentration of phospholipid was 125 μM.

3. Results

3.1. Selectivity of the peptide-induced stress

Each of the *E. coli* strains A–L (Table 1) contains a plasmid-borne genetic fusion of one of several promoter regions of *E. coli* stress responsive genes to a bioluminescent *luxCDABE* operon. Transcription initiated at the promoter region sequentially drives expression of the *lux* genes resulting in a bioluminescence response that requires the presence of ATP from the cytoplasm of viable cells. Therefore, in-

Table 1
Properties of the stress-sensitive strains of *E. coli*

	Gene type (::*lux*)/host	Strain	Stress	Inducer (conc.)	Ref.
A	*grpE′/**	TV1061	Protein folding	Ethanol (0.5 M)	[23]
B	*recA′/**	DPD2794	DNA damage	Mitomycin C (0.3 mM)	[29]
C	*katG′/**	DPD2511	Oxidative	H_2O_2 (15 mM)	[20]
D	*inaA′/**	DPD2146	Proton leakage	Salicylate (1 mM) CCCP (15 μM)	[27]
E	*lac/**	TV1048	Limited carbon source		[16]
F	*fabA/**	DPD1674	Fatty acid synthesis	Cerulenin (0.02 mM)	[33–35]
G	*osmY′/**	DPD2170	Hyperosmotic	Sucrose or NaCl (0.5 M)	[27]
H	*micF/**	DPD2191	Superoxide	Methyl viologen	TW
I	*micF/mar*$^-$	DPD2192	Superoxide	Methyl viologen	TW
J	*micF/sox*$^-$	DPD2193	Superoxide	Methyl viologen	TW
K	*micF/rob*$^-$	DPD2194	Superoxide	Methyl viologen	TW
L	*osmY/rob*$^-$	DPD2220	Superoxide	Methyl viologen	TW

TW, this work as described in Section 2.

creased transcription initiation, due to stress responsive regulation of gene expression, leads to increased transcription of the *lux* reporter and hence increased bioluminescence.

The growth inhibitory effect of sub-lethal concentration of PxB on the *lac-lux E. coli* strain E (TV1048) and of *osmY-lux* strain G (DPD2170) are compared in Fig. 1. The change in turbidity and luminescence of the *lac-lux E. coli* strain E follow a similar time-course during the early growth phase. This is expected because the luminescence increase is due to an increased cumulative expression of *lac-*

Table 2
Luminescence response from the stress sensitive strains A–K of *E. coli* to peptides and other additives

Peptide	Strains A–F	G	H	I	J	K	L
1. PxB	−	+	+	+	+	−	+
2. NP	−	ws	ws	ws	ws	−	*
3. Colistin	−	+	+	+	+	−	*
4. Colistin+NP	−	+	+	+	+	−	*
5. Cecropin A	−	+	+	+	+	−	*
6. Cecropin B	−	+	+	+	+	−	*
7. Magainin 1	*	+	−	*	*	*	*
8. Magainin 1+NP	*	ws	−	*	*	*	*
9. Magainin 2	*	+	−	*	*	*	*
10. Magainin 2+NP	*	ws	−	*	*	*	*
11. Mastoparan X	*	ws	−	*	*	−	*
12. Mastoparan X+NP	*	+	+	+	+	−	*
13. Mastoparan 17+NP	*	−	−	*	*	*	*
14. Gramicidin A+NP	−	−	−	*	*	*	*
16. Valinomycin+NP	*	−	−	*	*	*	*
17. Gentamicin+NP	*	−	−	*	*	*	*
18. Bacitracin+NP	*	−	−	*	*	*	*
19. CCCP	−(A,B,C,E,F)/+D	−	−	−	−	ws	*
20. Polylysine	*	−	−	*	*	*	*
21. Protamine	*	−	−	*	*	*	*
22. NaCl	−	+	+	+	+	ws	+
23. Sucrose	−	+	ws	ws	ws	ws	ws

Strains A–L (Table 1). More than 2-times increase in the luminescence is indicated by +. Less than 2-times increase above the background is indicated as a weak signal (ws). No detectable luminescence increase is shown as (−). *, not characterized.

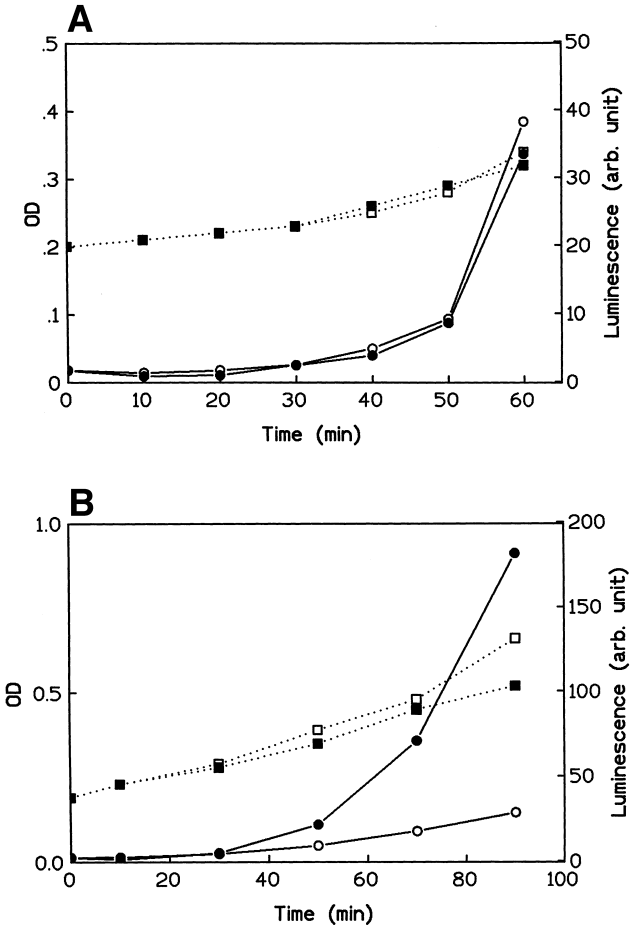

Fig. 1. Effect of PxB on the time-course of growth of: (A) *lac-lux* containing TV1048 strain E, or (B) *osmY-lux* containing DPD2170 *E. coli* strain G. The changes were monitored as turbidity (squares, dotted line), or the luminescence increase (circles, full line) in the absence (open symbols) or presence (closed symbols) of 0.25 μM PxB. Note that the values of the *X*- and *Y*-axes are different in A and B. Similar growth curves in the presence of sublethal 10 μM CCCP showed no effect in both cases (results not included, however see Tables 2 and 3).

lux genes with the increase in the cell population. In contrast, the *osmY-lux E. coli* strain G displayed an increased luminescence after 30 min of PxB treatment indicating an induction gene expression initiated at the *osmY* promoter. The TV1048 strain E is also well suited to monitor the effect of leakage and energy depletion in the cell because the luminescence reaction requires continuous supply of energy. In such assays of the short-term (<5 min) effects, the luminescence loss by CCCP above its MIC is pronounced [15]. This reduction in luminescence is expected if the ATP level required for the luminescence reaction is lowered due to a depletion of the proton gradient. However, even at 10 times above their minimum growth inhibitory concentrations, none of the peptides that we tested induced a rapid decrease (data not shown). Thus, proton leakage and loss of ATP is ruled out as the basis for the peptide induced antimicrobial stress (see also [15] and [16]).

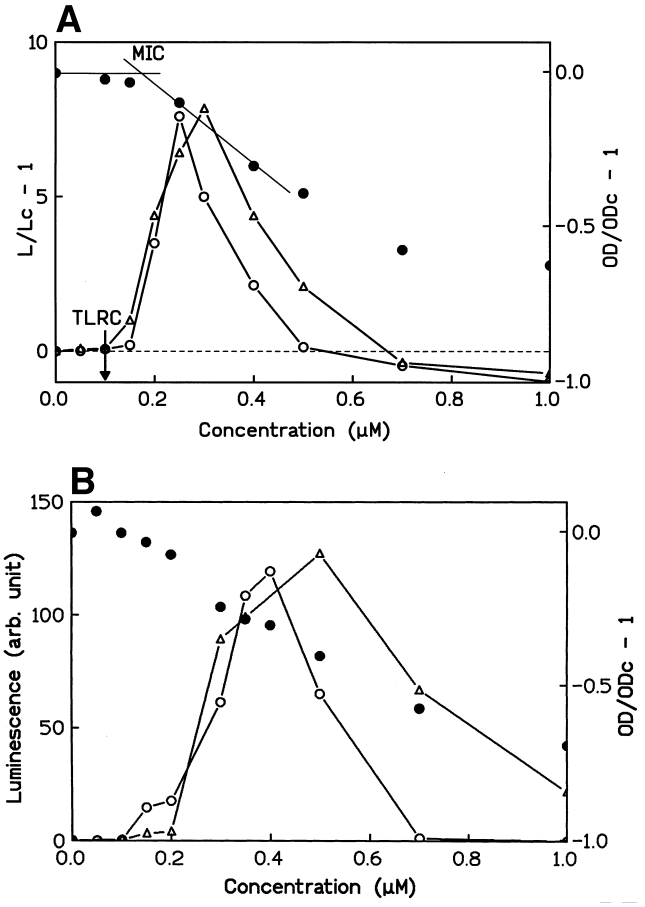

Fig. 2. The [PxB] (open circles) and [colistin] (triangles) dependence of the luminescence (open symbols) or turbidity (closed circles shown only for PxB) change after 60 min growth of (A) *osmY-lux* DPD2170 strain G or (B) *micF-lux* (*mar*$^+$*sox*$^+$*rob*$^+$) DPD2191 strain H. These results are parameterized (and summarized in Table 2) as: TLRC, threshold luminescence response concentration for the 60 min incubation; MIC, the minimum growth inhibitory concentration at 60 min. Note that the *Y*-scale is expressed in arbitrary units to emphasize the fact that in such cases the background luminescence is low, and therefore it is not meaningful to express the change as a ratio.

3.2. The transcription response is specific to osmY and micF fusions

The luminescence response protocol outlined in Fig. 1 was used to screen the effects of several antibacterial agents at MIC. As summarized in Table 2, the luminescence responsiveness of the A–L fusion strains to peptides and other forms of stresses shows a remarkable specificity for the structure of the peptide. Specific protocols and controls for these results are developed below. Antimicrobial peptides 1 through 12 and the hyperosmotic NaCl (#22) or sucrose (#23) induce luminescence response in the *osmY-lux* strain G. The *micF-lux* strain H exhibits virtually the same response profile, except that the response with magainins (#7–11) was not significant. Note that the stresses #1–6, 22 and 23 do not induce transcriptional responses from strains (A–F) that respond to macromolecular damage, depletion of the proton gradient, or oxidative damage. Similarly, the antibacterial stresses #14–21 do not show a response with strains G and H. Also, the proton-leakage responsive strain D does not respond to other stresses.

The response profiles for the *osmY-lux* strain G (Fig. 2A) and the *micF-lux strain* H (Fig. 2B) are biphasic. The maximum luminescence response in both strains is seen in the vicinity of the minimum growth inhibitory concentrations of polymyxin and colistin. Note that these plots are on linear ordinates, rather than on a logarithmic scale that is often used. With the use of a linear ordinate the concentration dependence of the response is significantly expanded in the region of the stress measured as the luminescence response.

The luminescence response from *micF-lux* fusion strain H to hyper- and hypoosmotic stress is shown in Fig. 3. The increase in luminescence is seen with hyperosmotic NaCl, but not with hypoosmotic stress. A modest growth inhibition is seen with both hypo- and hyperosmolar NaCl (data not shown). These results are similar to those induced by NaCl and sucrose in *osmY-lux* strain G [15,16], and suggest that the luminescence response of strain H from the induction of *micF* is primarily due to an increase in the osmolarity.

The expression of *micF* is controlled by three chromosomal regulatory genes, *marA*, *soxS* and *rob*, the products of which bind to the *micF* promoter region

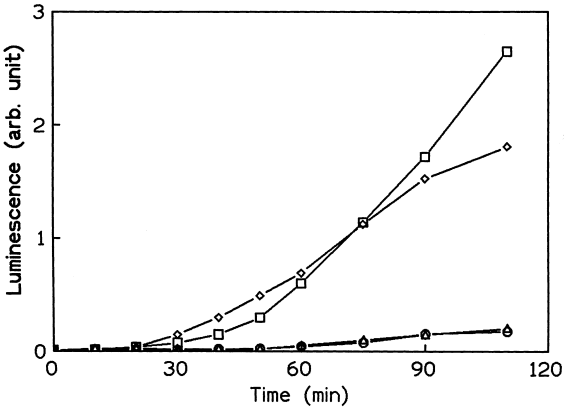

Fig. 3. The change in the luminescence during the growth of *micF-lux* DPD2191 strain H in the NaCl-free LB medium with 0 M (circles), 0.1 M (triangles), 0.3 M (squares) and 0.5 M (diamonds) NaCl.

[30,31,42–44]. As summarized in Table 2, deletion of *marA* in strain I (DPD2192) or *soxS* in strain J (DPD2193) did not alter the response profiles for the peptides or the hyperosmotic shock. On the other hand, inactivation of the *rob* gene in strain K led to the complete loss of the peptide induced luminescence and weakening of the hyperosmolarity induced luminescence. In contrast, the *osm*Y/*rob*$^-$ strain L was responsive to polymyxin demonstrating that functional *rob* is not required for the *osmY* response. These results clearly show that both *micF* and *osm*Y genes respond independently to the same set of stresses induced by hyperosmosis and the antibacterial peptides.

3.3. Antibacterial activity correlates with the ability of peptides to form contacts between phospholipid vesicles

Results in Table 3 show that polymyxin, colistin, cecropins, and magainins are growth inhibitory and induce the *osmY* transcriptional response. The MIC and the threshold luminescence response concentration (TLRC) change with the structure of the peptide. As compared in Fig. 4 (circles), MIC and TLRC are not only correlated with each other but also with the ability of the peptides to mediate phospholipid exchange between vesicles through stable contacts [38–40]. A direct and rapid exchange of monoanionic phospholipid through peptide contacts is monitored as a dequenching signal due to dilution of the probe phospholipid pyPG. The vesicles in contact do not

Table 3
Concentrations (μM) for inhibition of growth (MIC) and for threshold luminescence response (TLRC)[a] parameters for *osmY-lux* strain G of *E. coli*

No.	Peptide	TLRC	MIC	$FC_{1\%}$
1	Polymyxin B (PxB)	0.1	0.2	0.9
2	PxB-nonapeptide (NP)	30	40	10.2
3	Colistin	0.1	0.15	1.9
4	Colistin+NP	0.07	0.15	–
5	Cecropin A	0.07	0.1	0.3
6	Cecropin B	0.035	0.1	0.2
7	Magainin 1	5	12	7.8
8	Magainin 1+NP	5	10	–
9	Magainin 2	3	5	9.1
10	Magainin 2+NP	5	15	–
11	Mastoparan X	8	12	0.72
12	Mastoparan X+NP	3	4	1.5

[a]See Fig. 3A for the definition of parameters. TLRC, threshold luminescence response concentration; $FC_{1\%}$, the reciprocal of the signal (as % of the maximum change in the fluorescence, induced by 1 mol% peptide in the PyPG exchange assay); all measured parameters have an uncertainty of 20% on the same culture. Based on the repeated runs the uncertainty in these parameters is 30%. 10 μM NP, if present as the second component. N, no detectable effect.

exchange with excess vesicles. Thus the magnitude of the signal depends on the number of vesicles in contact, which increases with the concentration of the peptide. From the linear plot of the amplitude of the dequenching signal versus the peptide mol% (Fig. 5), we obtained the fractional change in the signal at 1 mol% peptide in the interface. The reciprocal of the amplitude is defined as $FC_{1\%}$ values summarized in Table 3. Since these values are obtained from a linear plot, they are directly related to the effectiveness of peptides to form vesicle–vesicle contacts.

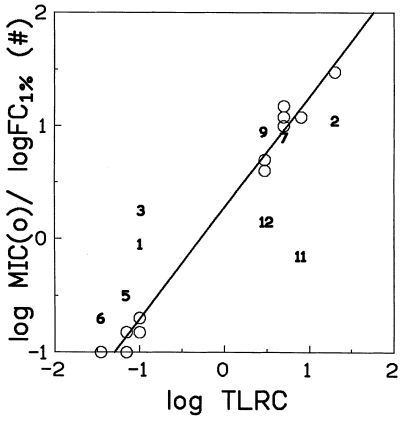

Fig. 4. Correlation between TLRC and MIC (open circles) or $FC_{1\%}$ (#). From results in Table 3.

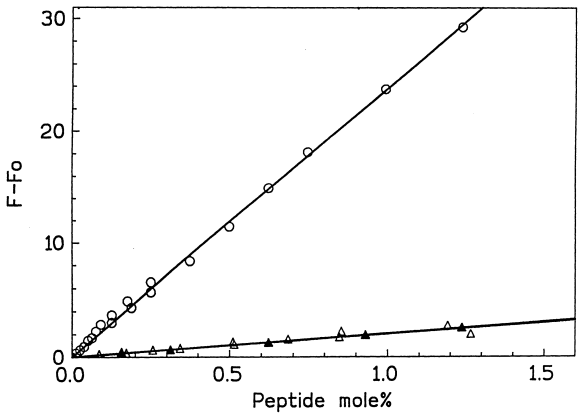

Fig. 5. The change in $(F-F_o)$ in the monomer fluorescence emission (at 398 nm with excitation at 345 nm) on the addition of varying amounts of cecropin B (circles), gramicidin (closed triangles), or PxB-nonapeptide (open triangles). Dequenching of pyPG/POPC (7:3) vesicles (2 μM total lipid) occurs as the pyrene probe is transferred through the peptide mediated contacts formed with a 125-fold excess of DMPG/POPG (7:3) vesicles. $FC_{1\%}$ value (Table 3) is defined as the reciprocal of the fractional change in the fluorescence in the presence of 1 mol% peptide added to the mixture of donor and acceptor vesicles.

Qualitatively, all antimicrobial peptides 1–12 promote exchange of PyPM, and a modest exchange is seen even with PxB-agarose (data not shown). No phospholipid exchange is seen with bacitracin, gentamicin, gramicidin, valinomycin and mastoparan-17 (data not shown). As shown in Fig. 4 (#), $FC_{1\%}$ trends with TLRC, that is at least qualitatively, the ability of PxB to form contacts correlates with growth inhibition and the transcriptional luminescence response. A possible origin of the scatter in the correlation of TLRC or MIC with $FC_{1\%}$ lies in the fact that the action of the peptide on whole cells involves at least two steps: disruption of the outer lipopolysaccharide layer, and the interaction of the peptide with the lipidic components surrounding the periplasmic space. The $FC_{1\%}$ values provide only a measure of the contact formation between phospholipid vesicles.

Several controls were also designed to identify the primary locus of action of the cationic antibacterial peptides on the basis of the hypothesis that the peptides could form stable contacts between the phospholipid interfaces of the bacterial periplasmic space.

For example the luminescence response from the *osmY* strain G is induced by PxB-agarose (results not shown), where the peptide is bound to agarose bead. Since PxB-agarose also has antibacterial activity [7,47,48], we conclude that entry of PxB in the cytoplasm is not required.

NP, the truncated (2–10)-PxB nonapeptide, inhibits its growth at a 200-fold higher concentration (Fig. 6). Origin of the response from NP is probably not due to the presence of PxB as an impurity because the peak luminescence intensity with PxB is considerably larger. Also, HPLC analysis on a C18 reverse phase column showed no detectable ($<0.05\%$) amount of PxB as an impurity in the NP preparation. We should have been able to detect such an impurity because NP and PxB are well separated. NP binds to LPS and disrupts the outer membrane of Gram-negative organisms with virtually the same efficacy as PxB, and NP makes the cytoplasmic membrane accessible to other solutes that do not otherwise cross the outer membrane [3,36,45,46]. Based on these reports, we rule out disruption of the outer membrane as a sufficient condition for the *osmY* or *micF* transcription, although results described next suggest that such a disruption of the outer layer is a necessary condition for the antimicrobial effect.

Ionophores like gramicidin A or valinomycin did not inhibit growth of the *osmY-lux* strain G unless 10 μM NP is also added to the growth medium (data not shown). This is because these peptide do not cross the outer membrane [49] unless NP disrupts the outer membrane and the peptide enters the periplasmic space. Note that 10 μM NP shows virtually no growth inhibition, nor any *osm*Y response (Fig. 6). NP had little or no effect on the *osm*Y response induced by peptides that induce the *osmY* response alone, and the *mic*F transcriptional response was not seen with a growth inhibitory combination of NP and ionophores (Table 2). For example, as shown in Fig. 7, 2 μM gramicidin A or 5 μM NP alone have no detectable effect on the time course of the background luminescence increase from growing *osmY-lux* strain G. In comparable experiments we did not see an increase in the luminescence at sub-lethal concentrations of gramicidin in the presence of NP. The fact that sub-lethal concentrations of gramicidin and NP showed no detectable increase in luminescence, suggests that the gramicidin-induced ion leakage through the cytoplasmic membrane does not induce the *osmY* transcriptional response. Virtually identical results are seen with valinomycin (not shown).

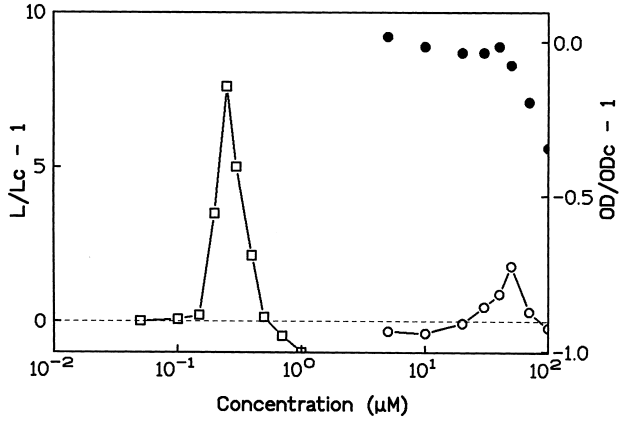

Fig. 6. The [NP] (open circles) and [PxB] (squares) dependence of the luminescence response after incubation for 60 min with growing *osmY-lux* DPD2170 strain G. The change in OD_{600} as a function of [NP] is shown as closed circles.

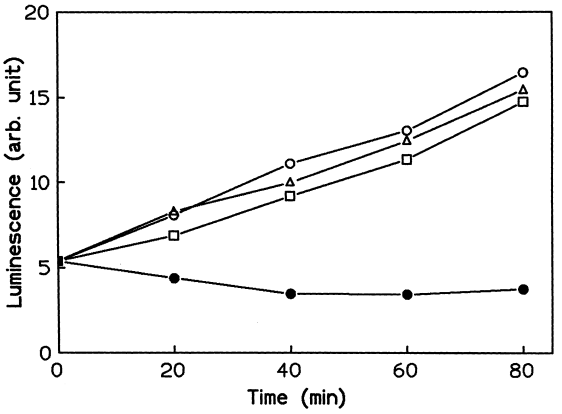

Fig. 7. The time dependence of the luminescence response with growth of *osmY-lux* DPD2170 strain: control without an additive (open circles), with 2 μM gramicidin A (triangles), with 5 μM NP (squares), or with 2 μM gramicidin A and 5 μM NP (closed circles). No luminescence increase was observed at the sub-lethal gramicidin+NP.

3.4. Adaptation and Resistance to PxB

An expectation of the hypothesis that phospholipid is the primary molecular target of PxB is that it may not readily succumb to some of the resistance mechanisms, such as the point mutation. Our results (not shown) affirm this expectation at several levels.

For example, as discussed later, certain PxB-resistant strains have modified Lipid A moiety, which presumably impairs PxB binding necessary for its uptake.

By HPLC and quantitative growth inhibition criteria, PxB is resistant to degradation under a variety of conditions (results not shown), although cecropin is degraded [16]. Resistance of PxB to degradation in the culture medium of several *E. coli* and *Pseudomonas* species permitted adaptation studies. Growth inhibition of the alginate+ strain by PxB suggests that the mucoid layer [50] does not prevent the action of PxB. Long term exposure of TV1048 *E. coli* strain and several *P. aeruginosa* (ATCC 10145 (type strain) and 27853; FRD1 (aliginate+), FRD1003 (alginate−)) or *P. putida* (ATCC33015) to PxB did not yield any stable growth. In agreement with earlier reports [51–53], only one strain of *P. aeruginosa* (PA10145 ATCC), showed a slow adaptation on PxB-gradient plates or by successive transfers to a medium containing 2–4 times MIC of PxB. In this strain cross-protection against 30 μM PxB was induced within 3 h in PA10145 cultures exposed to 100 μM NP. However, transfer of the 0.5 mM PxB adapted culture to a PxB-free medium restored the original PxB sensitivity, monitored as MIC, in less than 5 generation times. The metabolic profile (measured with the kit from Biolog) and the fatty acid composition of the original, adopted, and reverted strains were indistinguishable.

As a mechanism for adaptation, a point mutation in a protein receptor can render it ineffective against a drug. We searched for PxB-resistant strains in the EMS- or UV-mutagenized population of TV1048 and *P. aeruginosa* (ATCC 10145). Both of these strains did not yield a resistant strain. Within the limitation of the search, our negative results suggest that PxB resistance is not readily induced. Since the significance of these negative results is far-reaching, a more detailed search is warranted.

4. Discussion

Hyperosmotic stress and exposure to cationic peptide antibacterials selectively and independently promotes *micF* and *osmY* expression. The molecular functions of these gene products are not known; however, the regulatory circuits that control their expression are outlined in Fig. 8. Three general transcriptional regulatory factors that control several genes are implicated in stresses that concern us here. σ^{70} controls the osmoregulated ProU and ProP pumps, the proton-coupled transport proteins of the cytoplasmic membrane that control the solute efflux for the recovery from the hyperosmotic shrinkage of the cytoplasm. Also as a guide for future studies note that, as suggested to us by Dr. Patrick Brown (Stanford) on the basis of limited data, the expression pattern of *osmY* is similar to that of *osmC* followed by *osmE* and *Ybay*. Similarly, expression pattern of *rob* is similar to *Yfga*, followed by *Yabc*, *Yabb*, *Rlpb*, *Ychk* and *Nagc*. As discussed below, the *micF* expression controlled by *rob*, and the *osmY* expression controlled by σ^S are independently related to the action of antibacterial peptide or hyperosmotic stress.

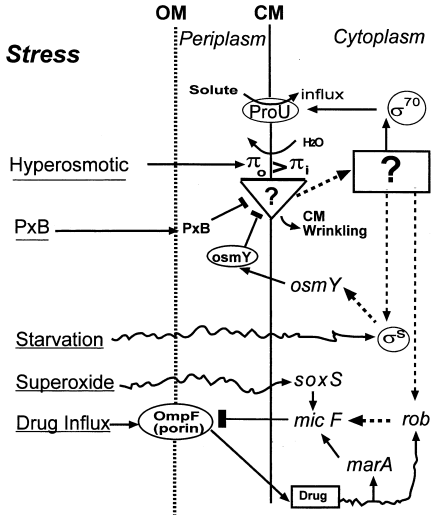

Fig. 8. Regulatory circuits in *E. coli* that respond to hyperosmotic shock, PxB, starvation, superoxide exposure, or drug influx. The effects of these stresses in the cytoplasm are regulated by the transcriptional regulators: σ^{70}, σ^S, or *rob*. Transcription of σ^S regulated *osmY* gene is promoted by starvation, hyperosmotic stress, as well as by PxB and related cationic peptide antibacterials. As suggested by results in the accompanying paper [60], these responses may be related to an event associated with the cytoplasmic membrane. Transcription of *micF*, which controls transcription of *ompF*, is regulated by *rob* and promoted by the same stresses. On the other hand, *proU* and *proP* transcription, which control the recovery from osmotic shrinkage, is regulated by σ^{70}.

4.1. Role of rob

Three different factors control *micF* transcription.

Expression of *micF-lux* fusion is induced by the redox cycling agent methyl viologen under the control of the *soxRS* regulatory circuit that respondes to the superoxide stress [20]. As expected, transcription of the *soxS*$^{-}$ strain J was not induced by methyl viologen. Expression of *micF* is also known to be induced by binding of the multiple antibiotic stress response regulator *marA* [42]. Over-expression of *rob*, *sox* or *marA* confers resistance to pegnazine methosulfate, as well as to antibiotics like chloramphenicol, tetracycline, nalidixic acid, or puromycin [30]. MicF produces an anti-sense RNA that inhibits expression of *ompF* encoding a porin. This circuit accounts for the regulation of drug influx. A distinct expression pattern for Rob versus SoxS and MarA is also reported for certain genes (*sodA*, *nfo*, *zwf, inaA*, *fumC*, *sodA*) [30].

Our results suggest that the detailed role of *rob*, *soxS* and *marA* gene activators is different because only *rob* is required for the response to the peptide or hyperosmotic stresses. Therefore, a role for the Rob protein is implicated in the response to the antimicrobial stress. Rob may also be involved in the *micF* response to hyperosmotic stress because a weakened response was observed in strain K lacking functional *rob*. A signal transduction pathway for the Rob protein has not been previously described. Rob is a DNA binding regulatory protein that binds to several promoters [54]. These results further suggest that the type of stress the cell sustains upon these treatments is distinct from previously known stresses. It is quite likely that in response to hyperosmolarity and the antibacterial stress *rob* may regulate expression of certain other proteins. However, the negative results in Table 2 clearly place certain constraints on such regulatory connections.

4.2. Role of *osmY*

OsmY is a hyperosmotically inducible gene that encodes a periplasmic protein in *E. coli* [55–58]. There are several stationary-phase responsive genes, and their products have a wide range of functions spread over several metabolic compartments. The *rpoS*-encoded sigma factor σ^S has been identified as a central regulator of many of these genes whose expression are stimulated by starvation or the onset of the stationary phase [55]. Transcription of *osmY* is directly regulated by σ^S, as well as several other regulators [56–58]. Also, σ^S expression is osmotically stimulated by a mechanism that operates at the levels of translation and σ^S protein turnover [58,59]. The transcription of *osmY* promoter occurs under all conditions; however, it is stimulated by hyperosmolarity, cell density, starvation, or other growth phase signals. Under these conditions, *osmY* expression is reduced but not eliminated in *rpoS* mutant [56]. Using a mutant that produces temperature sensitive σ^{70}, it has been shown that shifting this mutant strain to nonpermissive temperature did not affect osmotic activation of *osmY*, whereas σ^{70}-dependent osmoregulation through *proU* was strongly impaired [59].

The effect of the cationic antibacterials on the *osmY* expression implies that these peptides trigger the σ^S-dependent stress response, that is the treated cells go into stasis similar to that of late growth phase. This possibility is consistent with the fact that *E. coli* treated with the peptide antibacterials retain their proton gradient without cell lysis for more than 20 min [15]. A functional relationship between the OsmY protein in the periplasm and PxB is also suggested by the plasmolytic response seen during the first few seconds after hyperosmotic shock [60]. This is consistent with our hypothesis that both PxB and OsmY protein form contacts between the phospholipid interface surrounding the periplasmic space.

4.3. Significance of the lipid target and antibiotic resistance

Our studies [15,16] have uncovered a novel antibacterial mechanism based on a novel biophysical phenomenon, that is, the peptide-mediated intermembrane exchange of phospholipids [38–40]. It is intriguing that commonly used food preservatives, hyperosmolar NaCl and sucrose, exhibit the same profile of the transcriptional responses, i.e., the *osm*Y and *mic*F transcription is induced near the minimum inhibitory concentration for the growth by the antimicrobials. Also, the stasis is turned on by the peptides in the early growth phase [60]. As a fundamental process in bacterial physiology, control of stasis through physical and metabolic circuits is intrinsic in the functions of the *osmY* and *micF* gene products. By way of evaluating the broad significance of the finding we focus on the suggestion

that a lipid-based antibacterial mechanism may not succumb to resistance. However, as discussed below, the dialectic is polarized by numerous alternatives.

For its action, the antimicrobial must enter the periplasmic space, presumably by modifying the lipopolysaccharide layer. Since PxB interacts with the Lipid A portion of LPS, modification of this site may have a significant effect on the entry step. This is not inconsistent with the phospholipid target that does not succumb to point mutation. Resistance or a lack of susceptibility of Gram-negative organisms to PxB has been reported [60–69], including the adaptive resistance [51–53] possibly triggered by divalent cationic environment [70,71]. Lesion in the resistant strains appear to be localized in the LPS layer and associated with the expression of OprH gene. Modification of Lipid A of lipopolysaccharide has also been noted. Our results suggest that such adaptive changes cannot be induced in all strains, and are readily reversible. To explore the therapeutic and food preservative potential of antibacterial peptides, it will be necessary to find out whether the ability to modify LPS is present in all Gram-negatives, or if there are other mechanisms to breach the outer membrane.

To recapitulate, the evolutionary success of cationic peptide antimicrobials and their distribution in wide-ranging organisms implies distinct advantage of the underlying antimicrobial strategy. A better understanding of the antimicrobial mechanism of the peptides and hyperosmolar environment could ultimately provide a strategy for a better control and clinical management of infection in humans and animals. Widespread selection and ubiquity of antibacterial cationic peptides is possibly based on a strategy that is integrated with the physiology of stasis. Bacteria in stasis induced by the antimicrobials are likely to be cleared by the immune defense system in the host. During stasis, bacteria are not likely to loose their LPS, which minimizes the risk of septic shock. Since the peptide antimicrobials do not appear to enter the cytoplasm for their action, they may not be susceptible to drug efflux resistance. Similarly, inactivation of peptides by degradation would also require a long-term evolutionary solution. Obviously, some organisms do contain enzymes that modify Lipid A, which prevents the binding of PxB. Such an adoptive response must have a cost associated with it, because not all Gram-negatives seem to posses the adaptive ability.

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