ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2004, p. 3260–3267 Vol. 48, No. 9
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.9.3260–3267.2004

Genome-Wide Transcriptional Profiling of the *Escherichia coli* Response to a Proline-Rich Antimicrobial Peptide

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Received 10 February 2004/Returned for modification 8 March 2004/Accepted 3 May 2004

Most antimicrobial peptides (AMPs) impair the viability of target bacteria by permeabilizing bacterial membranes. However, the proline-rich AMPs have been shown to kill susceptible organisms without causing significant membrane perturbation and may act by inhibiting the activity of bacterial targets. To gain initial insight into the events that follow interaction of a proline-rich peptide with bacterial cells, we used DNA macroarray technology to monitor transcriptional alterations of *Escherichia coli* in response to challenge with a subinhibitory concentration of the proline-rich Bac7(1-35). Substantial changes in the expression levels of 70 bacterial genes from various functional categories were detected. Among these, 26 genes showed decreased expression, while 44 genes, including genes that are potentially involved in bacterial resistance to antimicrobials, showed increased expression. The generation of a transcriptional response under the experimental conditions used is consistent with the ability of Bac7(1-35) to interact with bacterial components and affect biological processes in this organism.

Antimicrobial peptides (AMPs) are components of the innate antimicrobial defenses of virtually all eukaryotic organisms (3, 18, 50). Nature's AMPs show an impressive variety of sequences (http://www.bbcm.units.it/~tossi/pag1.htm) and exhibit a number of different secondary structures. Those AMPs with β-sheet conformations stabilized by two or three disulfide bonds (denoted protegrins and defensins, respectively) and those with α-helical structures (e.g., LL-37, CRAMP, and BMAP-28) are most common in mammals (28, 47, 49). Other mammalian AMPs are stabilized by one disulfide bond (dodecapeptides) or form extended structures (e.g., the proline-rich PR-39 and Bac7 and the tryptophan-rich indolicidin) (14). Most AMPs share a cationic and amphipathic character that is thought to facilitate interaction with and insertion into the anionic microbial membranes, leading to disruption of membrane integrity (40). This lytic mechanism may result in inactivation of bacteria, fungi, enveloped viruses, and parasites (40). Peptides belonging to the proline-rich class, however, may act by a different mechanism (9, 15). These peptides are characterized by repeated proline-containing motifs and are active predominantly against gram-negative organisms. Investigations of the mode of action of the proline-rich apidaecins, pyrrhocoricin, and PR-39, isolated from the lymph fluid of honeybees, from *Pyrrhocoris apterus*, and from porcine neutrophils, respectively, have shown that these peptides kill responsive bacteria through a nonlytic mechanism(s), probably by inhibiting the activity of intracellular molecular targets (9, 15). Moreover, the ability of PR-39 to bind mammalian proteins containing Src homology 3 domains (5) and to induce the expression of specific mammalian genes (13) suggests it also has the capability to interact with host cell components and to signal biological responses.

Recent studies indicate that AMPs at nonlethal concentrations can act as environmental stimuli to elicit selected bacterial responses. For instance, treatment of *Escherichia coli* with the insect α-helical peptides cecropins at concentrations below MICs induces a hyperosmotic stress response in viable bacteria by activating the promoter of the hyperosmotic stress gene *osmY* (33, 34). In addition, microarray technology-based studies have shown that incubation of *E. coli* with a sublethal concentration of cecropin A causes selective alterations in the bacterial gene transcript profile (22), suggesting that this peptide could interact with microbial targets to evoke nonlethal responses in viable bacteria. Furthermore, the expression of various resistance genes has been found to be modulated in *Salmonella enterica* serovar Typhimurium (2) and *Pseudomonas aeruginosa* (29) in response to protegrin-1 (PG-1), LL-37, indolicidin, and other AMPs. These results highlight the contribution of genomic and proteomic methods to the elucidation of potential adaptation and/or resistance mechanisms of bacteria to AMPs.

In the present study, we examined the potential of a proline-rich AMP of 35 residues, denoted Bac7(1-35), to induce a transcriptional response in *E. coli*. Bac7(1-35) corresponds to the 35-residue N-terminal region of Bac7, which is a 60-residue AMP isolated from bovine neutrophils that selectively targets gram-negative bacteria (41). Bac7(1-35) retains the antimicrobial activity of Bac7 and impairs the viability of target bacteria without causing significant perturbation of the integrity of the bacterial membranes (42). This finding provides evidence for a nonlytic killing mechanism and suggests that the events that lead to bacterial death may be triggered by the binding of Bac7(1-35) to a selected molecular target(s).

To gain insight into the events that follow interaction of

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TABLE 1. Amino acid sequences of peptides used in this study

Peptide	Sequence	MIC (μM)[a]
Bac7(1-35)	RRIRPRPPRLPRPRPRPLPFPRPGPRPIPRPLPFP	10
Bac7(5-35)	PRPPRLPRPRPRPLPFPRPGPRPIPRPLPFP	>32
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVP RTES	12
PG-1	RGGRLCYCRRRFCVCVGR-NH_2[b]	1

[a] For *E. coli* MG1655.
[b] NH_2, C-terminal amidation.

Bac7(1-35) with target bacteria, we investigated here the ability of a sublethal concentration of Bac7(1-35) to stimulate a response in *E. coli* in terms of selective alteration of bacterial gene expression, by using DNA macroarray technology. The transcriptional profile obtained in response to Bac7(1-35) was analyzed and compared with that obtained with a peptide with a sequence corresponding to the 5-35 sequence of Bac7(1-35), denoted Bac7(5-35), which lacks the one to four N-terminal residues (RRIR) of Bac7(1-35) and is much less effective against *E. coli* and other gram-negative bacteria (42). We show that Bac7(1-35) causes selective alterations in *E. coli* gene transcription, including up-regulation of genes that are potentially involved in bacterial resistance. Conversely, Bac7(5-35) does not cause genomic responses in this strain.

MATERIALS AND METHODS

The sequences of Bac7(1-35), Bac7(5-35), LL-37, and PG-1 are shown in Table 1. All of these peptides were synthesized with a Milligen 9050 automated synthesizer (Applied Biosystems) with 9-fluorenylmethoxy carbonyl chemistry. Polymyxin B (PB) was from Sigma.

Bacterial strain and growth conditions. *E. coli* MG1655 was obtained from the *E. coli* Genetic Stock Center (http://cgsc.biology.yale.edu). Bacterial cultures were grown at 37°C in Luria-Bertani (LB) broth with constant aeration. Overnight cultures were diluted 1,000-fold in LB broth and grown at 37°C to an optical density at 600 nm between 0.05 and 0.06, corresponding to 3×10^7 CFU/ml.

Antimicrobial activity. Activity against *E. coli* MG1655 was determined as the MIC by a microdilution susceptibility assay, in 96-well plates. The experiments were run as previously described (41), except that assays were carried out in LB broth with a bacterial concentration of 3×10^7 CFU/ml, i.e., the same bacterial concentration used in the macroarray analysis. For bactericidal assays, bacteria (3×10^7 CFU/ml) were incubated at 37°C for 15 to 120 min in the absence or the presence of each peptide concentration. Serial dilutions of the bacterial cultures were then plated onto LB plates for CFU counts to assess the numbers of viable bacteria.

RNA isolation. Total RNA was isolated from *E. coli* MG1655 as suggested by the macroarray manufacturer (Sigma-Genosys Biotechnologies, Inc.). Bacterial cell pellets were resuspended in 10 mM sodium acetate (pH 4.2) containing 0.3 M sucrose and 0.5 M EDTA and then lysed in 10 mM sodium acetate (pH 4.2) containing 2% sodium dodecyl sulfate (SDS). Cell lysates were extracted three times with phenol buffered with 0.1 M citrate (pH 4.3) at 65°C, once with phenol-chloroform and once with chloroform. To remove contaminating genomic DNA, samples were treated with RNase-free DNase I (Amersham), followed by proteinase K (Sigma) digestion, phenol-chloroform and chloroform extractions, and precipitation with ethanol. Purified RNA was resuspended in water and quantified spectrophotometrically at 260 nm.

Generation of labeled cDNA from cultures of *E. coli* MG1655. Overnight cultures of *E. coli* MG1655 were diluted 1,000-fold in LB broth and grown at 37°C to an optical density at 600 nm between 0.05 and 0.06. Bacteria were then incubated at 37°C for 30 min in the absence or presence of 2.5 μM Bac7(1-35) or 2.5 μM Bac7(5-35). Total RNA was isolated (see "RNA isolation," above), and 1.5 μg was annealed to cDNA labeling primers (Sigma-Genosys Biotechnologies, Inc.) in a solution containing 50 mM Tris-HCl (pH 8.5), 8 mM $MgCl_2$, 50 mM KCl, and 1 mM dithiothreitol. After incubation at 70°C for 5 min, the mixture was cooled at room temperature for 10 min and then incubated for 3 h at 42°C in the presence of 0.33 mM dATP, 0.33 mM dGTP, 0.33 mM dTTP, 40 U of RNaseOUT (GibcoBRL), 50 U of AMV reverse transcriptase (Finnzymes), and 50 μCi of [α-^{32}P]dCTP. Unincorporated nucleotides were removed by Sephadex G-25 gel filtration chromatography.

Hybridization and analysis of gene arrays. Nylon membranes containing 4,290 open reading frame (ORF)-specific PCR products and representing a complete set of known or predicted protein coding sequences of *E. coli* MG1655 (Panorama *E. coli* Gene Arrays) were obtained from Sigma-Genosys Biotechnologies, Inc. Pair membranes were hybridized in each experiment with the cDNA synthesized from peptide-treated and untreated cells. Membranes were prehybridized for 60 min at 65°C in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA [pH 7.7]) in the presence of 2% SDS, Denhardt's reagent, and 100 μg of sheared salmon sperm DNA/ml. Hybridization with labeled cDNA (17×10^6 cpm) was carried out overnight at 65°C. Blots were then incubated twice for 5 min at room temperature and several times for 60 min at 65°C with 0.5× SSPE containing 0.2% SDS and finally subjected to autoradiography. The autoradiographic images were scanned, and the pixel densities of the resulting files were determined by Arrayvision version 7.0 software (Imaging Research, Inc.), which analyzes the intensity of each duplicate spot measured in arbitrary units. The background signal was determined for each group of genes by measuring the intensity of the nearest spot that did not contain DNA, selected from among 64 spots. This signal was then subtracted from the intensity of each DNA spot, and the corrected intensity was expressed as the percentage of total corrected DNA intensities. This procedure allowed comparison between filters independently of the total hybridization signal. The corrected intensities of duplicate spots were averaged, and the mean spot intensity of two independent experiments was calculated. The expression ratio for each gene was calculated as the ratio of spot intensities of treated and untreated cells (e.g., an expression ratio of 1 indicated an unchanged transcription level). A gene was considered significantly regulated when the expression ratio (ratio of treated cells/untreated cells) was $\geq$2.0 (up-regulated) or $\leq$0.5 (down-regulated). Only genes that showed at least a twofold increase in the expression level compared to the background signal in duplicate experiments were considered to be affected by antimicrobial peptide treatment.

Northern blot analysis. Total RNA (7 μg) was size separated on a 1% denaturing agarose gel, denatured by incubation in 8 mM NaOH for 20 min, and then transferred to a GeneScreen Plus nylon membrane (NEN Life Science) in a solution containing 3 M NaCl and 8 mM NaOH. Filters were prehybridized in ULTRAhyb solution (Ambion) for 30 min at 42°C and then hybridized for 6 to 24 h at 42°C with ^{32}P-labeled probes generated by PCR amplification of genomic DNA, as described below. After hybridization, membranes were incubated twice in 2× saline sodium citrate (1× SSC is 30 mM trisodium citrate and 0.3 M NaCl [pH 7.0]) in the presence of 0.1% SDS for 5 min and twice in 0.1× SSC with 0.1% SDS at 42°C for 15 min, then rinsed in 2× SSC, and finally exposed to X-ray film (Kodak). The intensity of each hybridization band was analyzed by densitometry. The background intensity was subtracted, and the net intensity of each gene was determined relative to that of *rrlH*. For each condition, the induction (*n*-fold) compared to that of the control culture was calculated. Genes were considered significantly induced or repressed when the treated/untreated cell ratio was $\geq$2.0 or $\leq$0.5, respectively.

Generation of gene-specific radiolabeled probes. Gene-specific probes for *secG*, *proP*, *proV*, *obgE*, *vacB*, *basS*, *ompT, malT*, *malK*, and *glpK* were generated by PCR amplification of *E. coli* MG1655 genomic DNA with *E. coli* PCR primers (Sigma-Genosys Biotechnologies, Inc.). Genomic DNA was purified from bacterial cultures as previously described (7). PCR amplification was performed with a total volume of 25 μl, including 0.6 U of Amplitaq Gold DNA polymerase (Perkin Elmer), 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, a 1 μM concentration of each sense and antisense primer pair, and 50 ng of *E. coli* MG1655 genomic DNA. After 10 min at 94°C, 30 cycles were performed with the following program: 30 s at 94°C, 40 s at the primer-specific annealing temperature, and 1 min at 72°C. Amplified fragments were analyzed with 1.5% agarose gels and purified from agarose with a Genelute Gel extraction kit (Sigma). PCR products were ^{32}P labeled with a T7 QuickPrime kit (Amersham Biosciences).

RESULTS AND DISCUSSION

Antimicrobial activity of Bac7(1-35) and Bac7(5-35). The amino acid sequences of Bac7(1-35) and Bac7(5-35) are shown in Table 1. MICs for *E. coli* MG1655 were determined after overnight incubations of 3×10^7 CFU/ml with serial dilutions of each peptide. Bac7(1-35) was found to inhibit the growth of

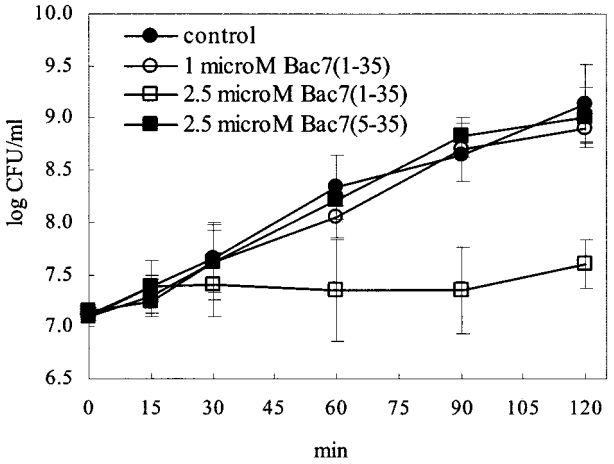

FIG. 1. Growth rate of *E. coli* MG1655 in the absence (control) or presence of 1 or 2.5 μM Bac7(1-35) or 2.5 μM Bac7(5-35). Data are means ± standard deviations of at least three experiments.

E. coli MG1655 with MICs of 10 μM. Conversely, Bac7(5-35) was ineffective for this strain at a MIC of 32 μM in four independent experiments. The sublethal concentration of Bac7(1-35) to be used in the macroarray analysis was worked out by determining the number of viable bacteria after treatment with Bac7(1-35) at 0.5 (results not shown), 1, and 2.5 μM (Fig. 1). The growth curve of bacterial cultures treated with up to 1 μM peptide was similar to that of untreated cultures, whereas at 2.5 μM (corresponding to a concentration which is fourfold lower than the MIC) Bac7(1-35) did not significantly affect the rate of bacterial growth during the first 30 min of incubation and caused a transient growth arrest at subsequent times (Fig. 1). A complete recovery was observed at 24 h (results not shown). The 2.5 μM concentration of Bac7(1-35) was considered for treatment of *E. coli* MG1655 with a sublethal peptide concentration. Bacterial cultures were treated in parallel with 2.5 μM Bac7(5-35) to assess the specificity of the response to Bac7(1-35). As anticipated by the MICs, the growth curve of *E. coli* MG1655 in the presence of 2.5 μM Bac7(5-35) was similar to that of untreated cultures at all times considered (Fig. 1).

Transcription profile of *E. coli* MG1655 treated with Bac7(1-35). The effect of 2.5 μM Bac7(1-35) on gene expression in *E. coli* MG1655 (3×10^7 CFU/ml) was examined by comparing the transcription patterns of bacterial cultures incubated for 30 min in the presence and absence of this peptide concentration. Genes whose relative expression ratios were ≥2 or ≤0.5 in two independent experiments were considered significantly altered.

Treatment with Bac7(1-35) caused substantial changes in the transcription of 70 bacterial genes. Among these, transcription of 26 genes was found to be repressed (Table 2) and transcription of 44 genes was induced (Table 3). The differential regulation of these genes was confirmed in two independent experiments, which produced similar results. Importantly, the expression profile of *E. coli* MG1655 exposed to 2.5 μM Bac7(5-35) was similar to that of untreated bacterial cultures (two independent experiments with similar results; data not shown), indicating that the positively charged cluster of residues at the N terminus is important both for the antimicrobial effect and for the induction of a genomic response.

TABLE 2. *E. coli* genes whose relative expression levels decrease after treatment with Bac7(1-35)

Gene	b No.[a]	Product or function[b]	Functional group	Ratio
aceE	0114	Pyruvate dehydrogenase component (E1) of pyruvate dehydrogenase	Energy metabolism	0.48
aceF	0115	Dihydrolipoamide acetyltransferase component (E2) of pyruvate dehydrogenase	Energy metabolism	0.35
aspA	4139	L-Aspartate ammonia lyase (L-aspartase)	Central intermediary metabolism	0.29
cyoE	0428	Cytochrome *o* ubiquinol oxidase C subunit	Energy metabolism	0.41
fecA	4291	Iron(III) dicitrate transport protein FecA precursor	Transport and binding proteins	0.45
gapA	1779	Glyceraldehyde 3-phosphate dehydrogenase A	Energy metabolism	0.28
glpK	3926	Glycerol kinase	Central intermediary metabolism	0.25
lamB	4036	Maltoporin, maltose high-affinity uptake system	Transport and binding proteins	0.24
lpdA	0116	Dihydrolipoamide dehydrogenase component (E3) of pyruvate dehydrogenase	Energy metabolism	0.45
malE	4034	Periplasmic maltose-binding protein	Transport and binding proteins	0.07
malF	4033	Maltose transport inner-membrane protein	Transport and binding proteins	0.25
malK	4035	Cytoplasmic membrane protein for maltose uptake	Transport and binding proteins	0.16
malM	4037	Maltose operon periplasmic protein	Carbon compound catabolism	0.31
malT	3418	Positive regulator of *mal* regulon	Transcription, RNA processing and degradation	0.43
rbsB	3751	Periplasmic ribose-binding protein precursor	Transport and binding proteins	0.20
rbsC	3750	High-affinity ribose transport protein	Transport and binding proteins	0.32
rbsD	3748	High-affinity ribose transport protein	Transport and binding proteins	0.43
sdaB	2797	L-Serine dehydratase 2 (L-serine deaminase 2)	Amino acid biosynthesis and metabolism	0.46
sodA	3908	Manganese superoxide dismutase	Cell processes (including adaptation and protection)	0.26
yfiA	2597	Protein Y, associated with 30S ribosomal subunit	Transcription, RNA processing and degradation	0.31
ygjQ	3086	Hypothetical 25.5-kDa protein	Hypothetical, unclassified, unknown	0.48
ygjT	3088	Hypothetical 35.8-kDa protein, putative membrane transport or efflux protein	Hypothetical, unclassified, unknown	0.39
yidA	3697	Hypothetical 29.7-kDa protein	Hypothetical, unclassified, unknown	0.23
yieJ	3717	Hypothetical 22.5-kDa protein	Hypothetical, unclassified, unknown	0.48
yijI	3948	Hypothetical 11.8-kDa protein	Hypothetical, unclassified, unknown	0.33
yrfD	3395	Hypothetical 30.0-kDa protein	Hypothetical, unclassified, unknown	0.16

[a] b number, unique identifier for *E. coli* genes.
[b] Based on EcoCyc database (24).

TABLE 3. *E. coli* genes whose relative expression levels increase after treatment with Bac7(1-35)

Gene	b No.[a]	Product (or function)[b]	Functional group	Ratio
amiC	2817	Putative cell wall amidase	Cell structure	2.9
basR	4113	Transcriptional regulatory protein BasR/PmrA	Transcription, RNA processing and degradation	2.0
basS	4112	Sensor protein BasS/PmrB	Transcription, RNA processing and degradation	15.7
betT	0314	High-affinity choline transport protein	Transport and binding proteins	2.7
bglF	3722	Beta-glucoside PTS permease	Transport and binding proteins	2.5
dnaK	0014	DnaK protein (heat shock protein 70)	Cell processes (including adaptation and protection)	2.6
fabF	1095	3-Oxoacyl-[acyl-carrier-protein] synthase II	Fatty acid and phospholipid metabolism	3.4
fimD	4317	Export and assembly of type 1 fimbrial subunits	Cell structure	3.0
hns	1237	DNA-binding protein (histone-like protein Hlp-II)	Translation, posttranslational modification	6.1
infB	3168	Protein chain initiation factor 2	Translation, posttranslational modification	3.0
insA_3	0275	Insertion element IS*1* protein InsA	Phage, transposon, or plasmid	2.1
insA_6	3444	Insertion element IS*1* protein InsA	Phage, transposon, or plasmid	2.4
insB_2	0264	Insertion element IS*1* protein InsB	Phage, transposon, or plasmid	2.1
intB	4271	Prophage P4 integrase	Phage, transposon, or plasmid	7.1
iscU	2529	Putative iron-sulfur cluster formation protein	Hypothetical, unclassified, unknown	2.1
lpxB	0182	Lipid-A-disaccharide synthase	Cell structure	2.2
metG	2114	Methionyl-tRNA synthetase	Translation, posttranslational modification	4.0
nagE	0679	*N*-Acetylglucosamine PTS permease	Transport and binding proteins	2.7
nusA	3169	L Factor	Transcription, RNA processing and degradation	6.4
obgE	3183	Putative DNA-binding GTPase	Hypothetical, unclassified, unknown	3.5
ompT	0565	Protease VII precursor	Cell structure	3.1
proP	4111	Proline-betaine transporter (proline porter II)	Transport and binding proteins	2.1
proV	2677	ATP-binding component of glycine betaine transporter	Transport and binding proteins	3.1
proW	2678	Integral membrane component of glycine betaine transporter	Transport and binding proteins	2.1
proX	2679	Periplasmic binding component of glycine betaine transporter	Transport and binding proteins	2.5
rplJ	3985	50S ribosomal subunit protein L10	Translation, posttranslational modification	2.3
rplM	3231	50S ribosomal subunit protein L13	Translation, posttranslational modification	2.1
rpmB	3637	50S ribosomal subunit protein L28	Translation, posttranslational modification	2.3
rpmE	3936	50S ribosomal protein L31	Translation, posttranslational modification	3.1
rpsO	3165	30S ribosomal subunit protein S15	Translation, posttranslational modification	2.3
secG	3175	Protein-export membrane protein	Transport and binding proteins	2.0
sstT	3089	Na^+/serine (threonine) symporter	Putative transport proteins	2.1
trmD	2607	tRNA (guanine-7) methyltransferase	Translation, posttranslational modification	2.1
vacB	4179	Exoribonuclease R	Hypothetical, unclassified, unknown	3.1
yafT	0217	Hypothetical 29.6-kDa protein	Hypothetical, unclassified, unknown	4.8
yceD	1088	Hypothetical 19.3-kDa protein	Hypothetical, unclassified, unknown	2.2
ydhR	1667	Hypothetical 11.3-kDa protein	Hypothetical, unclassified, unknown	2.9
yffS	2450	Hypothetical 31.0-kDa protein	Hypothetical, unclassified, unknown	2.3
yfiM	2586	Hypothetical 9.9-kDa protein	Hypothetical, unclassified, unknown	2.3
yhbC	3170	Hypothetical 16.8-kDa protein	Hypothetical, unclassified, unknown	3.1
yi21_5	3044	IS*2* hypothetical 13.4-kDa protein	Phage, transposon, or plasmid	2.8
yi52_4	1331	IS*5* hypothetical 39.3-kDa protein	Phage, transposon, or plasmid	4.7
yjeB	4178	Hypothetical 15.6-kDa protein	Hypothetical, unclassified, unknown	4.3
yodB	1974	Putative cytochrome	Hypothetical, unclassified, unknown	2.0

[a] b number, unique identifier for *E. coli* genes.
[b] Based on EcoCyc database (24). PTS, phosphotransferase system; IS, insertion element.

Among the genes that showed altered expression levels after treatment with Bac7(1-35), 73% corresponded to genes with known functions, 21% matched ORFs with unknown functions, and 6% corresponded to ORFs for which a function may be postulated by sequence homology comparison. Gene members of several operons (Table 2 and 3) were regulated in a coordinated fashion, supporting the congruency of the results. These included all genes of the *basRS*, *proVWX*, *malK-lamB-malM*, and *pdhR-aceEF-lpdA* operons; *infB*, *nusA*, and *yhbC* in the *metY-yhbC-nusA-infB* transcription unit; *malE* and *malF* in the *malEFG* transcription unit; and *rbsB*, *rbsC*, and *rbsD* in the *rbsDACBK* transcription unit.

To validate the results obtained by macroarray analysis, the expression levels of 10 genes picked up in response to Bac7(1-35), i.e., *secG*, *proP*, *proV*, *obgE*, *vacB*, *basS*, *ompT*, *malT*, *malK*, and *glpK* (Tables 2 and 3), were also examined by Northern analysis of RNA from treated and untreated cultures. These genes were selected from distinct functional categories. The results confirmed the regulatory trend observed with macroarray hybridization. Specifically, *malT*, *malK*, and *glpK* genes (Fig. 2 and Table 2) were strongly down-regulated in the presence of 2.5 μM Bac7(1-35), whereas the expression of *secG*, *proP*, *proV*, *obgE*, *vacB* (Fig. 2 and Table 3), as well as expression of *basS* and *ompT* (Fig. 3 and Table 3) was markedly induced. Similar results were obtained after exposing bacterial cultures to 1 μM Bac7(1-35) (Fig. 4), a peptide concentration that yields a growth curve comparable to that of untreated bacterial cultures.

Significantly, Northern blot analysis of RNA from bacterial cultures treated with Bac7(5-35) did not detect changes in the levels of these transcripts, compared with untreated cultures (results are shown for *basS* and *ompT*) (Fig. 3), in keeping with the macroarray hybridization pattern.

Bac7(1-35) affects the expression of a number of genes with diverse functions. Genes whose relative expression levels decreased in response to Bac7(1-35) are reported in Table 2. Most of these genes are involved in cell metabolism; among these, four different operons were significantly repressed. Two

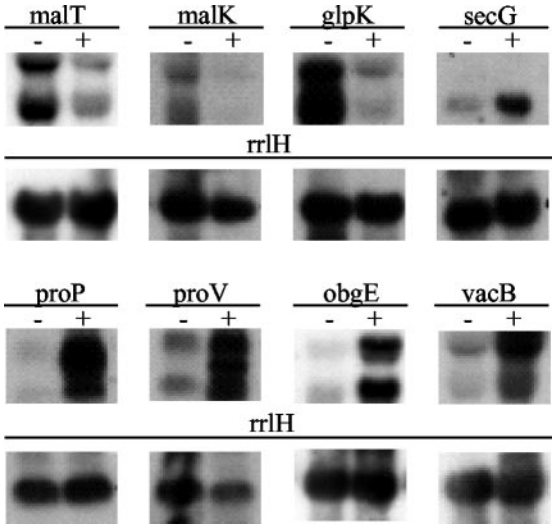

FIG. 2. Northern blot analyses of total RNA extracted from *E. coli* MG1655 after 30 min of incubation in the absence (−) or presence (+) of 2.5 μM Bac7(1-35). Blots were hybridized with gene-specific probes, as indicated. Hybridization with the *rrlH* probe served as a control for the amount and quality of RNA loaded in each lane.

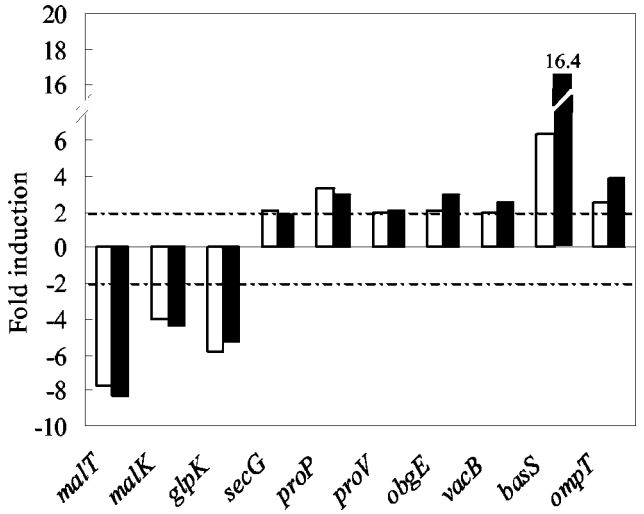

FIG. 4. Northern blot analysis of RNA extracted from *E. coli* MG1655 exposed to 1 μM (open bars) and 2.5 μM (filled bars) Bac7(1-35) for 30 min. The expression levels of the indicated genes were determined as described in Materials and Methods and are reported as levels of induction (*n*-fold) in peptide-treated cultures relative to that in untreated bacteria. Data representative of two independent experiments with similar results are shown.

of the operons encode the components of the maltose transport system MalKFGE that belongs to the ATP-binding cassette superfamily of transporters (4). Specifically, all gene members of the *malK-lamB-malM* operon and the first two genes of the *malE-malF-malG* operon were strongly repressed (repression ratio, 0.07 to 0.31). A downshift in the expression of *malG* was noted in both experiments, but the repression ratio (0.7; data not shown) did not reach the threshold considered significant. Interestingly, *malT*, a gene that encodes a positive transcription regulator of the maltose transport system (38), was also repressed in response to treatment with Bac7(1-35) (Table 2), suggesting that the alteration in the expression of the *mal* operon genes may be secondary to *malT* repression.

The other clusters whose genes were negatively affected by Bac7(1-35) include the *rbsDACBK* and the *pdhR-aceE-aceF-lpdA* operons. Three of the *rbs* locus genes were more than 50% repressed. Of these, *rbsB* and *rbsC* encode two subunits of the ATP-binding cassette transporter (RbsABC), which is responsible for ribose uptake (35). The *rbsD* gene encodes a cytoplasmic protein that binds ribose and may facilitate influx of this sugar in *E. coli* (25). The *pdhR-aceE-aceF-lpdA* gene cluster encodes the components of the pyruvate dehydrogenase complex. Other metabolic genes that were negatively affected by Bac7(1-35) include *gapA*, encoding the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase, *aspA*, *glpK*, *cyoE*, and *sdaB*. All these metabolic genes, apart from *gapA*, *cyoE*, and *sdaB* (for which information is not available), show a consensus sequence for the catabolic activator protein cyclic AMP (cAMP)-cAMP receptor protein (CRP) in their promoter regions (24). The requirement of cAMP-CRP for activation of *glpK* (48), *malE-malF-malG* (37), *malK-lamB-malM* (36), *malT* (4), *pdhR-aceE-aceF-lpdA* (21), and *aspA* (16) is also supported by the results of promoter binding and promoter activity assays. It is worth noting that although the expression of the *crp* gene coding for cAMP-CRP was only moderately altered in response to Bac7(1-35) (repression ratio, 0.65; results not shown), this slight repression correlates with the observed down-regulation of metabolic genes, also including *malT*, which codes for the main regulator of maltose genes.

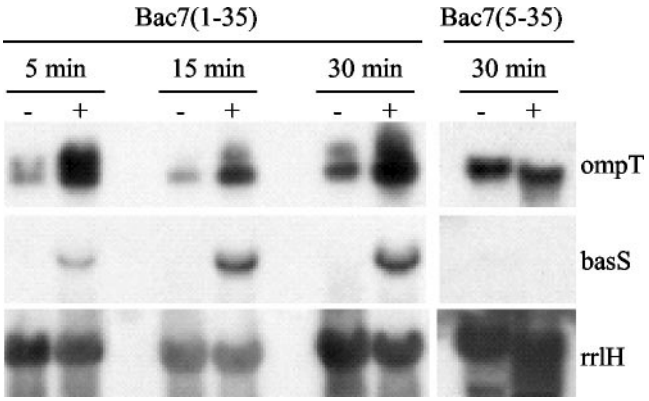

FIG. 3. Northern blot analyses of *basS* and *ompT* gene expression. Total RNA was extracted from *E. coli* MG1655 after incubation of bacteria in the presence (+) or absence (−) of 2.5 μM peptide for the indicated times. Blots were hybridized with *basS*-, *ompT*-, or *rrlH*-specific cDNA probes.

In the group of activated genes with known functions (Table 3), the *proU* operon and *proP* and *betT* genes are involved in the adaptive response of *E. coli* to osmotic stress. Specifically, *proV*, *proW*, and *proX*, which are encoded by the *proU* operon, are components of the ABC high-affinity transport system involved in the uptake of osmoprotectant glycine betaine and proline during osmotic stress (8). The *proP* and *betT* genes encode osmotically induced proteins involved in the transport of proline and betaine (30) and choline (26), respectively. The *proP* and *proU* genes, as well as *osmY*, a gene that was slightly

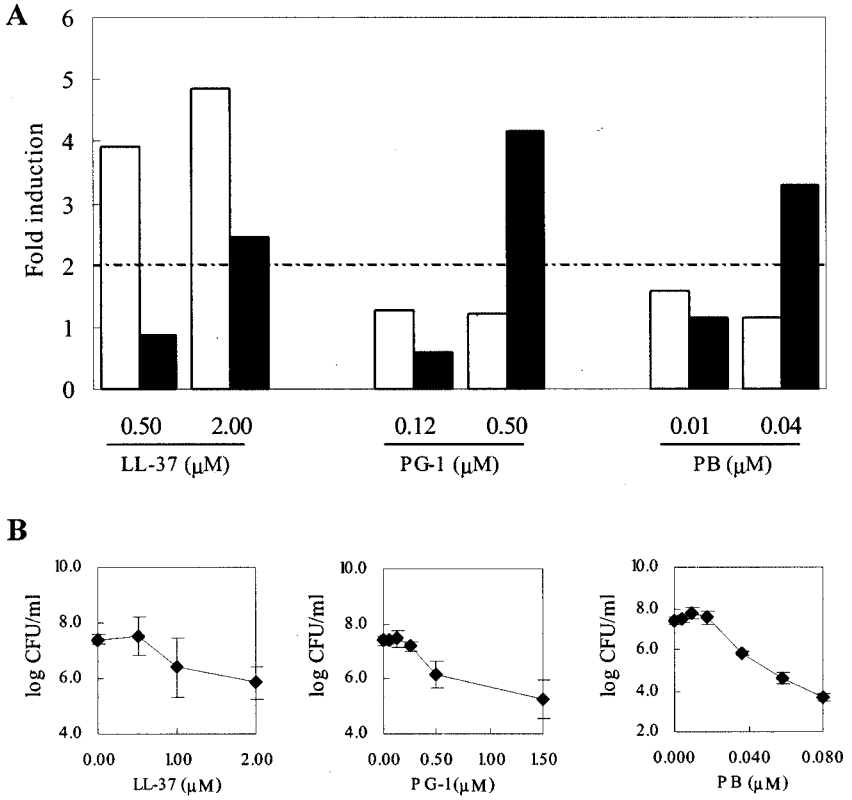

FIG. 5. (A) Induction of *basS* and *ompT* gene expression in response to LL-37, PG-1, and PB. Total RNA was extracted from *E. coli* MG1655 exposed for 15 min to each peptide at the indicated concentrations. Blots were hybridized with *basS*-, *ompT*-, and *rrlH*-specific cDNA probes. The expression levels of *basS* (open bars) and *ompT* (filled bars) were determined as described in Materials and Methods and reported as levels of induction (*n*-fold) in peptide-treated cultures relative to those in cultures of untreated bacteria. Data representative of two independent experiments with similar results are shown. (B) Activity of LL-37, PG-1, and PB against *E. coli* MG1655. Bacterial cultures were incubated for 15 min at 37°C in the presence of increasing amounts of each peptide, and the numbers of CFU per milliliter were determined. Data are means ± standard deviations of at least three experiments.

induced by Bac7(1-35) (induction ratio of 1.53; data not reported), are regulated by σ^s, a master regulator that is encoded by the *rpoS* gene and governs expression of stationary phase-induced and osmotically regulated genes in *E. coli* (19, 20). The transcription of the *rpoS* gene was not changed under our experimental conditions. This may be explained by the fact that the σ^s protein accumulates as a consequence of increased protein translation and protein stability under certain stress conditions, including hyperosmolarity (27, 31). Furthermore, high levels of this protein were detected in *S. enterica* serovar Typhimurium in response to PB, PG-1, and other AMPs (2).

The *hns* gene encodes the histone-like protein H-NS and was among the genes with the highest ratio of induction (6.1). H-NS is a major constituent of the nucleoid of *E. coli* and is involved in the negative regulation of gene expression in response to environmental changes and stress conditions (11). *hns* expression is subjected to autorepression to enable the cell to maintain a constant H-NS/DNA ratio (1). Our results indicate that this control mechanism is impaired by Bac7(1-35) treatment. The ensuing increase in the concentration of H-NS may result in drastic loss of cell viability, as suggested by overexpression studies of *hns* in *E. coli* (43). In this respect, *hns* may be one candidate effector of the antibacterial action of Bac7(1-35).

The group of positively regulated genes also included those encoding the BasR-BasS two-component regulatory system and OmpT (Table 3). *basS* showed the highest expression (induction ratio, 15.7) among all induced genes. This gene codes for a sensor inner membrane protein that, in response to external signals, phosphorylates and activates a *basR*-encoded transcription factor (32). BasR and BasS share approximately 85% amino acid sequence identity with the two-component regulatory system PmrA-PmrB present in *S. enterica* serovar Typhimurium. Activation of this system in *S. enterica* serovar Typhimurium in response to environmental stimuli leads to covalent modification that decreases the negative charge of lipopolysaccharide, thus reducing the affinity of this molecule for cationic antimicrobial peptides and proteins (17, 39). It is interesting in this respect that the PmrA-PmrB system is activated in *P. aeruginosa* in response to AMPs such as PB, indolicidin, and LL-37 (29).

OmpT is an outer membrane protease and may be an essential factor for the virulence of *E. coli* strains involved in complicated urinary tract disease (12, 23). This protease is responsible for bacterial resistance to AMPs (46), since it inactivates cationic peptides by a specific cleavage between two basic residues (10).

The response of *E. coli* to Bac7(1-35) in terms of expression

of *basS* and *ompT* was investigated further and was compared to that of other AMPs under similar experimental conditions.

Time course analysis of the expression of *basS* and *ompT* genes (Fig. 3) revealed basal *ompT* expression levels in *E. coli* MG1655 and in the absence of added stimuli. A fast and marked induction of both *basS* and *ompT* was detected in the presence of 2.5 μM Bac7(1-35) (Fig. 3). Conversely, bacterial incubation for up to 30 min in the presence of 2.5 μM Bac7(5-35) did not alter the expression of these genes (Fig. 3), in keeping with the macroarray results.

To investigate whether an increase in the levels of these genes is a common response of *E. coli* MG1655 to AMPs, three structurally different cationic AMPs with lytic properties, i.e., the β-hairpin peptide PG-1, the α-helical LL-37, and the cyclic PB (44, 45, 49), were comparatively analyzed for their capacity to up-regulate *basS* and *ompT*. Bacterial cultures were incubated for 15 min with a sublethal and a lethal (1 log CFU decrease after 15 min of incubation) (Fig. 5B) concentration of each peptide. LL-37 induced significant up-regulation of *basS*, whereas PG-1 and PB had little effect on this gene at either peptide concentration (Fig. 5A). All three peptides caused >2-fold increases in *ompT* expression at concentrations that inhibited bacterial growth but were poorly effective on this gene at sublethal concentrations (Fig. 5A), possibly suggesting that *ompT* is up-regulated in response to physical damage caused by these molecules. Taken together, these results indicate that the lytic peptides are far less efficient than Bac7(1-35) in modulating the expression of *ompT* and that only α-helical LL-37, among these peptides, shares with Bac7(1-35) the ability to up-regulate *basS*. The latter effect may not be simply related to the α-helical structure of LL-37, since the expression levels of *basS* and *ompT* were unchanged following a 10-min incubation of *E. coli* MG1655 with both sublethal and lethal concentrations of the α-helical peptide cecropin A (22). These individual differences within peptides with similar structural features and/or antimicrobial properties thus likely reflect different and as-yet poorly understood modes of interaction with prokaryotic cells.

Conclusions. This study is the first contribution to the definition of the transcriptional profile of a prokaryotic organism following treatment with a proline-rich peptide. We have shown that Bac7(1-35) at concentrations below its MIC for *E. coli* MG1655 induces a complex transcriptional response that involves changes in the expression of a selected set of functionally distinct groups of genes. Some alterations are consistent with activation of stress adaptation mechanisms; some, particularly those indicating reduced metabolic activities, may be connected to the antibacterial action of Bac7(1-35). Several other changes, including the induction of genes involved in protein synthesis (Table 3), are not easily understood in this context. A comparative analysis of the gene transcript profile of *E. coli* MG1655 treated with Bac7(1-35) and gene transcript profiles determined for *E. coli* MG1655 during transient or permanent growth arrest (6) indicates that most genes whose expression is altered on exposure to Bac7(1-35) (Tables 2 and 3) are unaffected or show an inverse regulatory trend during growth arrest (6). For instance, the vast majority of genes encoding the transcription and translation apparatus down-regulates under transient arrest or stationary-phase conditions (6), whereas various ribosomal genes and other genes involved in transcription and translation are up-regulated in the presence of Bac7(1-35) (Table 3). Transport and binding protein-encoding genes that are induced by Bac7(1-35) (Table 3) are unaffected or repressed (6), and those repressed by Bac7(1-35) (Table 2) are up-regulated (6) during growth arrest. An exception seems to be represented by genes involved in aerobic respiratory metabolism. Most genes in this category down-regulate during growth arrest (6), and some of these are repressed after exposure to Bac7(1-35) (Table 2).

To unravel the complexity of this response, it will be necessary to extend this analysis and characterize the bacterial gene transcript profile at various incubation times and peptide concentrations, to better refine the peptide-specific response, to discern temporal patterns of transcription, and to achieve a more complete understanding of the sequential events that follow interaction of Bac7(1-35) with bacterial cells.

We have shown that the positively charged cluster (RRIR) at the N terminus of Bac7(1-35) is required for induction of the transcriptional response. Prior studies indicated that removal of these residues from the peptide sequence substantially decreased the microbicidal activity (42). Overall, these results suggest that the charged N-terminal region of Bac7(1-35) is essential to enable this peptide to interact with bacterial components and interfere with biological processes. It remains to be clarified whether these residues are needed for the initial interaction with the bacterial surface, for specific binding to internal targets, or for both these processes.

ACKNOWLEDGMENTS

We are grateful to A. Tossi from the BBCM Department of the University of Trieste for reading the manuscript.

This work was supported by grants from the Italian Ministry for University and Research (P.R.I.N. Cofin. 2002), the Interuniversity Consortium for Biotechnology (CIB), and Regione FVG.

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